

High noon at Kyoto

An agreement between the United States and developing countries, brokered by Japan, could ensure a successful outcome for the forthcoming conference of the United Nations climate convention at Kyoto.

Next month, the United Nations environmental bandwagon rolls into the ancient Japanese city of Kyoto for 10 days of talks that are intended to culminate in a new legally binding treaty to reduce anthropogenic greenhouse-gas emissions. If agreed, this treaty will be a tribute as much to the hard work of scientists as to the long nights put in by negotiators at Kyoto. Indeed, the prospects for the meeting would have been considerably dimmer without the 10-year efforts of the United Nations' body of climate scientists, the Intergovernmental Panel on Climate Change, and, in particular, its statement that the balance of evidence suggests anthropogenic climate change. But agreement at Kyoto hinges not on science — whose importance will re-emerge subsequently (see pages 225–226) — but on the willingness of governments to settle their differences.

So far, most have adopted predictable positions. The Group of 77 developing states wants the richer countries to make radical cuts to their greenhouse-gas emissions. This amounts to a 15 per cent reduction from 1990 levels by 2010. The United States has responded with a decidedly unradical target (stabilization of emissions to 1990 levels between 2008 and 2012) coupled to a raft of controversial conditions including a demand for major concessions from the developing countries. Member states of the European Union, meanwhile, are on the middle ground, proposing major cuts but without seeking developing-country concessions. Between the European Union and the United States lies Japan, with its own widely derided compromise target of a 5 per cent emissions reduction from 1990 levels by between 2008 and 2012.

Inevitably, the final outcome at Kyoto will disappoint many. But it is too soon to write off the meeting. Indeed, there are at least two good reasons not to be wholly pessimistic. The first concerns the host nation, the second, US insistence on 'meaningful participation' from developing countries.

Japan as broker

Japan has not had a good press as it prepares for its first major United Nations conference. An image has emerged of Japan as a reluctant host, eager to please, but not really up to the job of brokering an important international agreement.

Is Japan trying to please everyone? Undoubtedly to some extent. Japan's consensus-style political culture is steeped in the type of deal-making on which UN agreements are built. Its own emissions reduction proposal illustrates that it has a solid understanding of this process. Japanese officials know that there will be no deal unless the United States can be brought on board. They also know that Europe is prepared to be 'flexible' on its proposed target, as well as on its opposition to emissions trading.

Therefore, far from being a drawback, Japan's consensual approach is precisely what may salvage an agreement at Kyoto. Indeed, Japan's expertise in mediation potentially mirrors the diplomatic skills wielded in recent years by countries of Scandinavia and other 'small' north European countries. Also in Japan's favour is its choice of Britain's deputy prime minister, John Prescott, to chair informal talks between Europe and the United

States. Britain's Labour party enjoys close relations with the Clinton administration. And although Prescott may not have finger-tip command of international environment policy, his deal-making skills are beyond doubt.

What of developing-country participation? The United States wants 'meaningful participation' from major developing countries such as China, India, Brazil and Mexico. But developing countries are reluctant to reduce their greenhouse-gas emissions just yet. Many are still in the process of building an industrial base, and do not want to be forced to pay to correct a problem — anthropogenic climate change — caused mainly by others. Two scientific papers in this issue bear on the developing-country question (pages 267–270 and 270–273). They will prove controversial, and at least one is open to misinterpretation (see pages 227–228).

Per-capita targets

To many governments, and many more environmentalists, the US requirement of developing-country participation effectively ends any hopes that remain of meaningful agreement at Kyoto. But to others it represents an opportunity, not a threat (see pages 215–220). It is no secret that when the time comes, many developing countries would much rather base emissions targets on a single per-capita calculation rather than merely a percentage increase or reduction over 1990 levels.

The idea of a per-capita level is at present viewed with some scepticism in Europe and the United States. But if the United States sticks to its insistence on early emissions reductions from developing countries, it will need to give something in return. US support for a per-capita convergence of emissions could be that something.

US officials have not so far ruled out the idea of global greenhouse-gas emissions converging to a per-capita value. But they may need more evidence that enough developing countries support the idea. They also need more evidence that developing countries will agree to early emissions reductions in return for US support for per-capita emissions convergence. Until now, only the Africa group of countries has shown any enthusiasm. There is as yet no official word from India, nor from China, despite the latter's known preference for a per-capita system. Both countries may want to wait until the final hours of Kyoto before they reveal their hand. In the meantime, support from environmentalists would not go amiss.

Most developing countries and environmentalists have so far resolutely opposed embarking on a discussion of per-capita emissions. They agree with the per-capita principle, but disagree with the idea of early developing-country participation which, they rightly point out, violates the terms of the climate convention. But if Kyoto is to be saved from turning into a damp squib, they would be well advised to change their minds.

Clearly, the host nation has much to do. As a result of domestic interministerial negotiations, Japan's own proposal incorporates the developing-country desire for emissions to be reduced to a per-capita level. If the Japanese broker an agreement on this issue between the United States and developing countries, they will have fully justified their role as host. □



NATO science programme feels the heat over funds and goals

[PARIS & MONTREAL] The North Atlantic Treaty Organization (NATO) science programme plunged into crisis last week when the NATO council, prompted by Canada, failed to approve next year's budget for the programme. The crisis stems from growing pressures on NATO finances caused by its enlargement in Eastern Europe and internal conflict about the goals of the science programme — indeed, whether it is still required at all.

The programme was set up in 1957 as part of NATO's bid to strengthen security and cooperation among its 16 member states. It spends around \$40 million a year on a series of basic and applied research programmes that have attracted the support of some of the best scientists in NATO countries. NATO fellowships, Collaborative Research Grants and Advanced Study Institutes have become familiar and well-respected.

The crisis has been precipitated by the costs of the enlargement of NATO, which the Pentagon estimates at \$27 to \$35 billion over 12 years. This includes a \$5 billion increase in NATO's own budget over 10 years, on top of the current \$1.8 billion that it already spends on administration and other activities each year.

The science programme is part of NATO's civil budget, which is much smaller than its military budget. One senior NATO

official says the programme is particularly vulnerable because funding comes from foreign ministries, for whom science is not a high priority.

The tensions came to a head earlier this month, when Canada unilaterally decided to stop its funding for the science programme. Rodney Moore, a spokesman for the Department of External Affairs, says the decision was taken not only for budgetary reasons but also because Canada felt that the programme was no longer needed to strengthen cooperation among NATO partners.

Cuts in the science programme are also favoured by the United States, Britain and



the Netherlands, whereas France and Spain would like funding to be maintained at current levels. There are also deep differences over strategy.

Over the past five years, the share of the science programme assigned to supporting cooperation with Eastern Europe and Russia has grown from 5 per cent to almost half. Many NATO countries, including Canada, the United States, France and Britain, would like to see this increase further — one of Canada's stated reasons for its withdrawal was what it claimed was lack of progress in orienting the programme to this goal. But this idea is opposed by smaller countries such as Portugal and Turkey, which would lose out.

Attempts to align the goals of the science programme more closely with diplomatic imperatives received strong support in a recent review of the programme by an independent panel, chaired by Roland Schmitt, a physicist and former head of General Electric's research centre in Schenectady, New York, and including seven other leading researchers. Collaboration with NATO's 24 'cooperation partner' countries in Eastern Europe and defence-related research should be priorities, the panel concluded.

Speaking before the North Atlantic Council in Brussels earlier this month, Schmitt said that the challenge of enlargement in terms of science was as great as that which led to the programme's creation in the first place. "Failure to respond at this moment in time will be tragic, while responding with vision will be seen as a historic response to a unique but transient opportunity," he said.

Science provided a relatively uncontroversial means of underpinning diplomatic goals and tackling security issues such as disarmament and the threat of biological weapons, Schmitt said. He added that NATO had an important role to play in preventing the decline of science in Eastern Europe by maintaining and creating international links. This would "enhance the security of Europe as a whole".

Defending the emphasis on "outreach", Schmitt said it was "with great reluctance" that the review panel recommended that money for this should come from cuts in the intra-alliance programme, given that the science budget would not be increased.

NATO officials hope that Canada can be persuaded to change its position, and that support for the science programme can be mustered. "Discussions are quite heated at the moment, but we hope to get consensus," says one official. **Declan Butler & David Spurgeon**

US carbon emissions forecast to rocket

[WASHINGTON] Carbon emissions in the United States will soar to a level more than one-third higher than that in 1990 by 2010 if current trends continue, according to new official projections. The projections indicate that it will be more difficult than previously forecast to return emissions even to their 1990 level by 2010, let alone to achieve a large reduction, as many countries are demanding should be agreed at the Kyoto meeting on climate change next month (see also page 209 and *Briefing*, page 215.)

The Energy Information Administration (EIA), an independent statistical agency inside the US Department of Energy, announced last week that, under current best projections of economic growth and energy prices, carbon emissions are forecast to exceed 1990 levels by 34 per cent by 2010 and by 45 per cent by 2020.

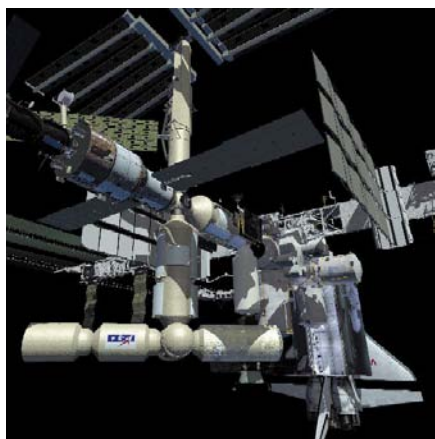
The projection for 2010 is five per cent higher than the agency estimated a year ago, chiefly because of continuing strong economic growth combined with lower

prices for energy, especially electricity. The new data suggest it would be even tougher than expected for the United States to meet the target of reducing greenhouse-gas emission to the 1990 level by 2008–2012, which it will take to the Kyoto meeting.

The Clinton administration has already identified the pending restructuring of the US electricity industry as a key opportunity for changes that will reduce carbon emissions. But the EIA projects that restructuring will actually increase emissions by cutting electricity costs, and therefore raising consumption. It will also slow the growth of renewable energy sources of electricity.

The EIA also points out that the 44-point Climate Change Action Plan, which the Clinton administration introduced in 1993 with the aim of stabilizing greenhouse-gas emissions at 1990 levels by the year 2000, has failed to have the desired effect, and expects emissions to overshoot that goal by 17 per cent.

Colin Macilwain



NASA space station chief resigns

[WASHINGTON] Wilbur Trafton, the senior official in charge of the US space station (above) and space shuttle programmes, announced his resignation last week after less than two years as the space agency's associate administrator for space flight.

Although he cited personal reasons, Trafton leaves at a time when the space station programme is under increasing financial pressure. With construction scheduled to begin in Earth orbit next June, the National Aeronautics and Space Administration (NASA) is \$200 million short of the amount it says it needs in 1998 to keep the project on track (see *Nature* 389, 530; 1997).

In testimony to Congress earlier this month, Trafton said the agency was consulting with the White House budget office on possible solutions to the funding problem. Without more money, he said, NASA would be forced to choose between three options: stretch out assembly of the station, which is now scheduled to be completed in 2003; delay the beginning of onboard research; or stop work on an 'interim control module' being built in case Russia fails to launch a key piece of station hardware by early 1999.

The threat of delaying onboard research is likely to upset the programme's critics in Congress. Although station 'utilization' is not scheduled to begin until January 2000, with the arrival of experiment racks to be placed inside the US laboratory module, limited research involving astronauts can begin as soon as the first permanent crew arrives in January 1999. Laboratory equipment fitted in place in November 1999 will allow additional experiments. But that early research capability may be in jeopardy unless NASA solves its imminent money problems.

The head of NASA's authorizing subcommittee in the House of Representatives, Dana Rohrabacher (Republican, California), praised Trafton as an "incredibly decent and capable man" who took the heat in Congress for bad decisions made by senior officials at NASA.

Tony Reichardt

Funding delay looms for EU Framework programme

[MUNICH] A delay in approving the European Union (EU)'s fifth five-year Framework programme of research (FP5), which it was hoped would begin towards the end of next year, looks increasingly likely. The result could be a gap in the allocation of European research funds when the current Framework programme runs out at end of 1998.

Neither the Council of Ministers, which represents the 15 member states, nor the European Parliament, which must reach a common position by February if delay is to be avoided, have resolved disagreements about the content and cost of FP5.

Meeting in Brussels last week, EU research ministers are reported to have made little progress towards the unanimous position they must adopt on FP5. Instead, they deferred decisions until after the parliament's position is clear.

The proposal submitted by the European Commission (EC), through which FP5 is funded, foresees 16 key actions. These would be organized into three 'thematic programmes' — the information society, the living world and ecosystem, and competitive and sustainable growth — and three 'horizontal programmes', on international cooperation, participation of small and medium-sized businesses, and improving human potential (IHP), which would cut across the thematic programmes.

The EC proposes a budget of ECU16.3 billion (US\$18.7 billion), including ECU1.47 billion for nuclear research under the separate Euratom treaty. This would keep the proportion of EU gross domestic product spent on research the same as for FP4.

Most research ministers last week confirmed support for an increase to five in the number of thematic programmes, so that life sciences, energy and environment would each be single programmes.

But some want more. Sweden, for example, continued to press for a thematic programme on social and economic research, Portugal for a single marine programme, and the Netherlands supports just the three thematic programmes originally proposed.

Ministers confirmed their opposition to EC proposals to reduce the role of member states in overseeing Framework programmes. The EC wants more autonomy to decide issues such as final short-lists of projects; at present, this requires formal approval from programme committee composed of representatives of member states.

FP4 has 18 programme committees. The EC proposes that FP5 should have only six, each with reduced responsibilities. It says

this would speed approval of grants. But ministers insisted the EC's activities should continue to be closely monitored. This issue will probably be discussed again at a special meeting in January.

Ministers also failed to reach a common position on how much FP5 should cost. Smaller and poorer countries, which benefit considerably from EU subsidies, support the EC proposal. But Germany, France and Britain, the EU's three biggest contributors, want to keep the cost down to ECU13.2 billion, the unadjusted cost of FP4.

Perhaps the biggest threat to FP5's timetable comes from the parliament, which must vote on its formal position in mid-December. Before then, the FP5 proposal's rapporteur, Godelieve Quisthoudt-Rowohl, a Christian Democrat from Germany, must rationalize 639 amendments received in response to the EC's proposal.

Amendments relate to both the content and the cost of the programme — the socialists want a total budget of ECU17.8 billion — and the way in which the budget should be divided. Together with the parliamentary committee on research, technological development and energy, Quisthoudt-Rowohl must prepare a draft likely to win parliamentary approval before mid-December.

Some amendments support a transfer of money from nuclear to non-nuclear energy research. Others want more to be given to the IHP programme, and for it to include a social and economic research programme.

The financing of the IHP programme, which has attracted broad political support, may prove controversial.

An amendment proposed by Quisthoudt-Rowohl herself would reduce by nearly one-fifth the money for IHP, reallocating it to life sciences. A further controversial amendment from Quisthoudt-Rowohl proposes opening IHP to non-European countries.

"With so many proposed amendments it is difficult to say exactly how the structure of the programme will look when we introduce it to the parliamentary plenary," says a spokesman for the research committee. He offers "slightly greater than evens chances" that agreement will be reached for mid-December.

Many observers now believe that the grand political aim of FP5, to concentrate resources on fewer scientific areas, is unlikely to be met. Virtually nothing in FP4 has been dropped in the commission's FP5 proposal, while discussions in council and parliament make it clear the list of programmes and key actions will grow.

Alison Abbott

Wellcome Trust to coordinate drive against malaria

[PARIS] Britain's Wellcome Trust has been given responsibility for coordinating the activities for the coming year of a recently created powerful forum to lead a new attack on malaria.

The Multilateral Initiative on Malaria (MIM) is a loose consortium of research and donor agencies, including the US National Institutes of Health, the UK Medical Research Council, the World Bank and United Nations agencies.

The third meeting of MIM, held last week in London, agreed formal commitments to a series of objectives. Disagreement fuelled by the different interests of the agencies involved had almost brought an earlier meeting in The Hague to a crisis (see *Nature* 388, 219; 1997).

MIM has now formally adopted proposals agreed in outline at The Hague, while the decision to make Wellcome responsible for coordination, for the first year at least, is widely seen as a means of depoliticizing the forum — the Wellcome Trust is perceived as relatively neutral and an 'honest broker'.

In another development, the meeting decided there was little point in developing new models of international cooperation when there was little new money available beyond the \$100 million a year currently available for malaria research worldwide.

As a result, "communication and advocacy" of the malaria problem emerged as the first priority. This was formally delegated to the Malaria Foundation, which is based in New York, but which is considering opening an office in Europe as well.

In a related move, the chief executives of the world's biggest pharmaceutical companies meet tomorrow (21 November) to consider a proposal supported by the World Health Organization, the Wellcome Trust and other organizations which is aimed at developing a private-sector-backed international structure to restart research into antimalarial drugs.

Declan Butler

Greenhouse emission cuts 'in China's own interests'



[LONDON] Leading climate scientists in China have concluded that the country's long-term economic and environmental interests would be best served by reducing greenhouse-gas emissions — even though they remain "considerably less pessimistic" about the effects of climate change than their counterparts in the United Nations climate panel.

"Half of China's motive for reducing carbon dioxide emissions is to protect the country from rising sea levels," says Ren Zhenqiu, a member of the China Academy of Meteorological Sciences. "The other half is to fulfil our obligations to countries like the United States, even if this is at the cost of giving up the benefits of global warming to China. This should be the basis of China's policy of reducing carbon dioxide emissions and participating in international negotiations."

Ren's comments appear in a three-volume report on Chinese climate research, an English summary of which was released on the Internet by the US Embassy in Beijing earlier this month (<http://www.redfish.com/USEmbassy-China/sandt/CLCHNG.HTM>). The volumes have been published over the past five years by 150 researchers from the Chinese Academy of Sciences and other agencies.

Although the three climate change volumes are written by scientists, not policy-makers, they are seen as an indication of how China's policy-makers are thinking. Chinese scientists recognize global warming has benefits as well as costs. The authors point out that China has experienced global warming before. Ren is quoted as saying that 4,000–8,000 years ago, China was 2–4 °C warmer than today. Sea levels were 1–3 metres higher, and there was 20–40 per cent more rainfall.

Although a warmer, wetter climate would benefit agriculture in some parts of China, the

authors recognize that increased greenhouse emissions would deplete the country's fossil-fuel base, as well as threatening coastal states.

But their reports also emphasize that for China, development has traditionally taken priority over environmental protection. This was also effectively the message contained in a speech last month in Beijing by Song Jian, a scientist who is also leader of the environment group in China's ruling State Council.

Song said China was committed to reducing greenhouse-gas emissions, but not if it meant depriving people of refrigerators "or those who live at temperatures of 40 °C of air-conditioning". He nevertheless indicated that China would cut emissions by increased use of renewable energy, afforestation and lower population growth, which he described as "the decisive guarantee of protecting the climate and environment". He hinted that China favoured reducing greenhouse-gas emissions on a per capita basis (see page 217).

One observer of China's environment policy says "Song Jian is a distinguished scientist as well as an important Chinese official, and one would do well to pay great attention to what he says."

Ehsan Masood



More motor vehicles in China means increasing carbon emissions — and blocked bicycle lanes.

MIKE HALL/AP

French agency agrees to publication of asbestos report

[PARIS] INSERM, the French national biomedical research agency, is to publish as a book a controversial report by an expert panel on the risks of asbestos. An earlier decision to make the report 'publicly available' was criticized on the grounds that it relegated the report to the status of 'grey' literature.

Claude Allègre, the research minister and an outspoken critic of the report, has strongly refuted allegations that formal publication of the report had been blocked by INSERM on the instructions of his

ministry (see *Nature* 389, 649; 1997). "Why would I have opposed distribution of a text of mediocre quality?" he asked at a recent press conference. INSERM has played down the allegations as "a storm in a teacup."

INSERM now claims that the eventual publication of the report had always been a possibility. But a letter sent last month by Jean Marimbert, head of the ministry's department of labour relations, to Claude Griscelli, director general of INSERM, states that during a meeting "INSERM

representatives stated their decision not to publish at INSERM Editions the final report."

The letter goes on to demand that INSERM reverse this decision. Relegating the report to 'grey' literature would "damage the scientific credibility of a study that was decisive for the public authorities," says the letter, and would strengthen the hand of those opposed to the ban on asbestos introduced following the release in July 1996 of the report's conclusions in their preliminary form.

D.B.

Bavarian universities set to gain autonomy

[MUNICH] Universities in Bavaria, Germany's largest state, will become the first in Germany to have explicit mechanisms for ensuring research excellence, competition for grants and budget autonomy, following the approval last month of a radical new university law.

Under the law, universities will in future be able to appoint professors, distribute their own budgets and open or close faculties without reference to the science ministry. They will also be able to introduce degrees directly equivalent to bachelors' and masters' degrees.

Bavaria's 10 universities will also have to compete for the first time for their share of the state budget for higher education. They will be judged not just on student numbers but also on research criteria, such as numbers of graduate students and the amount of external grant money received.

The monitoring role of the Bavarian ministry of education, science and culture will be taken over by an external advisory university board, known as the *Hochschulrat*. Each university will have its own *Hochschulrat*, consisting of three representatives from local industry and two independent academics whose appointments will require the approval of the science minister. It will have advisory powers only.

The creation of *Hochschulräte* has been



Herrmann: wanted stronger boards.

the most controversial aspect of the new law. The University of Basel in Switzerland became the first German-speaking university to introduce such a board in 1996: its board has full decision-making power. At least from an administrative point of view, the Basel model is regarded as a success; for example, the average time to appoint a professor has been reduced from two years to six months.

But there has not yet been a significant increase in the value of grants from industry. "Increasing the share of outside financing, which was 10 per cent or so of the total university budget in 1996, has top priority in the coming years," says Beat Münch, spokesman for the University of Basel.

The Bavarian *Hochschulräte* will not have decision-making powers. But they have still proved unpopular with those who fear that the heavy presence of industrialists will push universities too far towards applications-oriented research. Also, smaller universities fear that the best industrial representatives will serve on the boards of the big universities based in Munich, the Bavarian capital, and that they will be left with the second best.

But others are enthusiastic, although

Wolfgang Herrmann, president of Munich's Technical University — the most important university for science in Bavaria — says he would have preferred a board with decision-making powers, rather than one with only advisory and monitoring functions.

The Bavarian parliament is expected to give rapid approval to the bill and the boards will have to be in place by autumn next year. Other *Länder* in Germany have been discussing reform of their own university laws, but so far with little success. Although *Länder* hold full responsibility for universities, they have to work within a federal 'framework' law, intended to guarantee equal value to all academic degrees, regardless of the university awarding them.

Reform of this framework law is under way to allow competition between universities, which are creaking under the strain of student numbers — all students with a high-school leaving certificate have the right to a university education. But this would simultaneously destroy the concept of equivalence of degrees, which is one of the main reasons the long-discussed changes have still not been approved (see *Nature* 388, 820; 1997).

Meanwhile, Bavaria's conservative science minister, Hans Zehetmair, is pleased his extensive reform plans have been put into practice. "It was a balancing act, but we made it in the end," he says. **Quirin Schiermeier**

ALBERT SCHÄRGER

Lower budget deficit offers hope of research bonus in Canada

[MONTREAL] Canadian researchers are queuing to claim their share of a fiscal dividend which the Finance Minister, Paul Martin, expects from lowering the federal budget deficit.

Separate presentations to the Parliamentary Standing Committee on Finance have called for a doubling of the Medical Research Council (MRC) budget, a 60 per cent increase in Social Sciences and Humanities Research Council (SSHRC) funds, and a 50 per cent increase in the budget of the Natural Sciences and Engineering Research Council (NSERC).

Also, the Council for Health Research in Canada, a group of voluntary health agencies and research institutions, is aiming to alert government and the public to "Canada's weak investment in health research (which) contrasts sharply with the trends and priority given to research in several other G-7 countries".

Some scientists are optimistic of success, as Martin has said that half of any surplus would go to social spending and has also spoken positively about science, technology and innovation. But the government may not provide money in the councils' annual

budgets, as that would lock it into more or less permanent expenditure. The outcome will not be known for at least a month.

The Council for Health Research in Canada says expenditure on health research has fallen by about 10 per cent since 1990; US spending has risen more than 80 per cent in the same period. Canada's MRC allocates only C\$7.27 (US\$5.2) per capita for 1998, whereas the US National Institutes of Health allocate C\$49.8 per capita. The council recommends minimum increases in MRC's base budget of C\$60 million for each of the next four years, resulting in a doubling by 2002.

A submission by the Association of Universities and Colleges of Canada, the Canadian Association of University Teachers, the Canadian Consortium for Research, the Humanities and Social Sciences Federation of Canada and the Canadian Graduate Council recommends an increase of 50 per cent in federal funds for the granting councils over four years.

Last year, the government committed C\$800 million to a Canada Foundation for Innovation. The submission says that, with funding for this beginning in 1998, "investments in the granting councils in

subsequent years will be critically important if we are to take advantage of the enhanced research capacity".

The submission also calls as an "utmost priority" for 20 per cent of new granting council funds to be allocated to promote research careers. Thomas A. Brzustowski, president of NSERC, elaborated on this in asking for large increases in research subsidies totalling C\$160 million.

Brzustowski said the key to success in the knowledge-based economy was to prepare young people to match the world best in science and engineering, but this is not happening. Support for postgraduate students by NSERC, for example, has not changed significantly for five years.

The result is that many fail to develop to their full potential or go abroad, where they receive much higher levels of support. Brzustowski says that an increase of at least 25 per cent is needed in both the value and number of postgraduate scholarships.

He also called for a fourfold extension of NSERC's Undergraduate Student Research Awards in laboratories across Canada, and a 25 per cent increase in graduate and postdoctoral stipends. **David Spurgeon**

NIH to abandon young investigator grants

[WASHINGTON] Senior officials at the US National Institutes of Health (NIH) last week decided to end a special category of grants restricted to new investigators. From next year, all new grant applicants will compete in the larger, more competitive pool for standard grants.

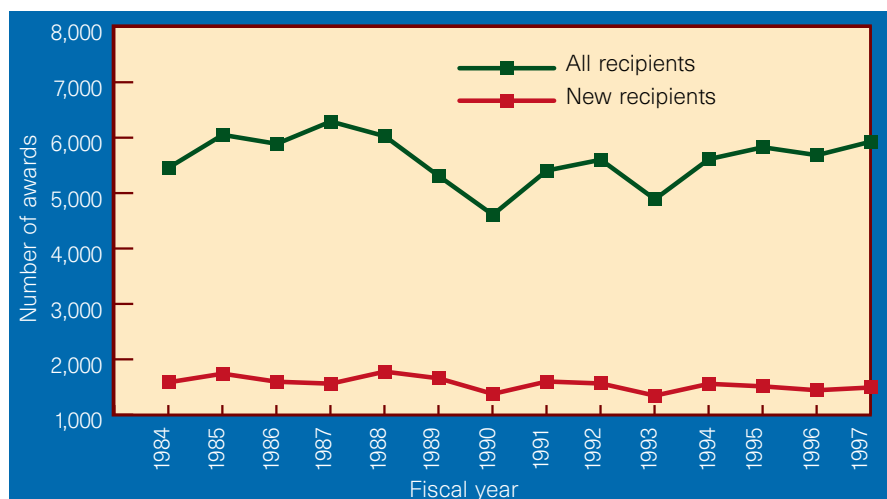
But by committing itself to maintaining the total number of new NIH investigators — 1,466 new investigators entered the NIH system in 1997, about one-third on new investigator grants (see figure) — the NIH has effectively promised to deliver up to \$300 million more in total grant funding in the year five years hence, when the last of the much less costly grants for new investigators expire.

"This is a really important signal about how strongly the institutes and [NIH director Harold] Varmus feel about the support of new investigators," says Wendy Baldwin, the deputy director of NIH's Office of Extramural Research. "This is going to cost us."

She adds that the change will modify a funding system that has inadvertently created a second class of financially constrained investigators. "We don't want two classes of new investigators. We want people to size their grants appropriate to the work they are proposing to do."

The grants to be phased out are called R29s, or 'First Independent Research Support and Transition' (FIRST) awards. They were established in 1987 to help new investigators make the transition from postdoctorate to independent investigator, and provide \$350,000 in direct money over five years, or \$70,000 a year.

About one-third of new investigators enter the NIH system on these grants. The



Recipients of NIH competing research project grants (R01 and R29), 1984–97.

Source: NIH

others enter directly into competition for standard R01 grants, which deliver on average \$138,000 a year in direct costs — about twice as much as the R29s.

At a meeting last Thursday (13 November), the heads of NIH's institutes voted unanimously to adopt a recommendation proposed last spring by an NIH working group on new investigators. This concluded that the small size of R29s was hampering young researchers more than access to a less competitive applicant pool was helping them (see *Nature* 387, 835; 1997).

The working group pointed out, for instance, that new investigators who were awarded R29s (except for those in the top 10 percentile) were significantly less successful — 6 to 8 per cent — in getting future grants than new investigators who entered the NIH

system on R01s.

Under the new policy, existing R29s will be funded throughout their current lives. But from next June, NIH will no longer accept R29 applications.

New investigators will apply for standard grants, with their applications flagged as coming from first-time applicants, and reviewers will be asked to "give new investigators a break", says Marvin Cassman, director of the National Institute of General Medical Sciences, who co-chaired the working group. For instance, new applicants will be granted "some leeway" in terms of preliminary data: "We want reviewers to give the same benefits to the new R01 applicants that they gave to the R29 applicants," Cassman says.

Baldwin says that she and Elvera Ehrenfeld, director of NIH's Center for Scientific Review (formerly the Division of Research Grants) will work with reviewers this winter to determine the best way for applications to be flagged. Something as simple as a stamp on the front could suffice for now, she says. The actual application form cannot be changed overnight — government procedures will prevent that happening for 18 months.

"Although the R29 certainly sounded like a good idea it just didn't work out all that well," says Cassman. "You can't conduct a reasonable research program on \$70,000."

NIH officials say that the extra \$300 million required to fund today's number of new investigators on more generous standard grants is a rough estimate that assumes that the costs of R01 projects proposed by new investigators will match those in the overall pool. It could be less if new applicants as a group come in with more modest grant proposals. But the number incorporates a commitment to maintaining an influx of new investigators sufficient to replace the annual 8–9 per cent attrition rate among principal investigators.

Meredith Wadman

Mixed experience with R29 'baby grants'

[WASHINGTON] The US National Institutes of Health (NIH) working group that reported last spring (see above) found a common complaint among R29 recipients it interviewed: funds were so paltry that they were forced to spend large amounts of time writing applications for other grants to meet their laboratory costs.

Jais Lingappa, 38, a cell biologist at the University of California, San Francisco, calls her R29 funding "untenable". She says that \$70,000 a year "barely covers my salary plus a small amount for equipment, and is not enough to hire anybody else to work with me".

As a result, says Lingappa, who landed her R29 this year, she is now considering applying for an R01 and giving up the smaller grant. "I certainly wish that [the NIH decision] had been instituted earlier."

Anecdotal evidence also suggests that those awarded R29s have been handicapped by the perception that they are 'baby grants' without the prestige of R01s. David Kupfer, chairman of psychiatry at the University of Pittsburgh School of Medicine, says that he used to encourage young scientists to apply for R29s.

But he stopped after watching a tenure committee deny a young neuroscientist partly because of the "stigma" of his R29 grant.

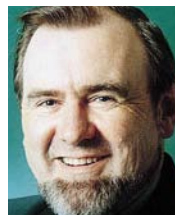
But others have had a different experience. George Prendergast, a 36-year-old molecular geneticist at the Wistar Institute in Philadelphia, was recently promoted to associate professor. Since receiving an R29 grant three years ago, he has applied for and received an additional \$1.1 million in funding from other organizations for his cancer research laboratory. "It certainly hasn't held us back," he says.

M. W.

Australia told that universities need greater competition

[CANBERRA] Competition among Australia's 36 public and two private universities is likely to intensify, with funds coming largely through students rather than directly from government, if a review of higher education is accepted by the government.

The review comes as higher education has barely settled down after restructuring initiated 10 years ago, and when it has been buffeted by heavy funding cuts. The review says there is "a compelling case for bolstering" research infrastructure, and urges that priorities for university research be "explicitly" based on national goals.



West: opposed to voucher proposal.

The seven-member review panel was set up last January after the former Education Minister, Amanda Vanstone, was accused of failing to lay out guidelines for universities while requiring them to maintain functions with resources effectively reduced by 10–20 per cent (see *Nature* 387, 222; 1997).

The wide-ranging review, chaired by Roderick West, a teacher of classics and retired principal of a private school, has set a goal of "universal access" for up to 95 per cent of school-leavers in a "seamless" system of universities and technical colleges. West says that he accepts universities are "hard-pressed" financially, but believes the system can cope with current funding levels if it becomes more flexible and open to market forces.

West wants to encourage an appreciation of 'Learning for Life' — the title of the first of the committee's two reports. Like other committee members, he favours granting students 'learning accounts' (or 'vouchers') to buy places on courses at institutions of their choice. He also talks of separating research funding from teaching.

The present system provides block grants to universities according to government-prescribed numbers of students and courses. Under the panel's proposals, universities would be freed from much government control and allowed to set their own fees. Postgraduates would be given scholarships transferable between universities.

David Kemp, Vanstone's successor after a recent cabinet reshuffle, reduced the political impact of the review by immediately declaring that the Coalition government would not approve a voucher scheme. A proposal for vouchers, which Kemp himself had proposed in opposition, was a major factor in the Coalition's failure to defeat the then Labor government in 1993.

Peter Pockley

Synchrotron delays 'put at risk' Europe's leading role

[PARIS] As Switzerland was last week giving its final approval to a planned 2.1-GeV Swiss Light Source, British and French scientists repeated calls for their respective governments to approve swiftly construction of proposed national synchrotron radiation sources in the two countries.

Scientists meeting at a two-day international conference in Paris on the use of synchrotron radiation in biology and medicine united behind a simple take-home message: modern synchrotron facilities are not a luxury, but an essential resource in modern biology. They warned that further delays may risk Europe losing its lead over Japan and the United States in this area.

British research administrators are scrutinizing future budgets to discover how to pay for a proposed 3-GeV synchrotron machine, Diamond. Their French colleagues face a different frustration: although the money has been promised by public funding agencies the government appears reluctant to give its approval to the 2.15-GeV Soleil, in particular because of political difficulties over the choice of a site.

There is broad consensus among researchers on the need for new synchrotrons in Europe. At the Paris meeting, those voicing support included Paul Williams, chairman and chief executive of the UK Central Laboratory of the Research Councils, Catherine Bréchnac, director-general of the French Centre National de la Recherche Scientifique (CNRS), Yannick d'Escatha, director-general of the French Atomic Energy Commission (CEA), and Stephen Bieri, chief executive officer of the Swiss Federal Institutes of Technology.

The four organizations signed an agreement to cooperate in the design of one another's machines, swapping technical expertise, sharing staff and cutting costs through economies of scale. A permanent board, with two people from each country, will coordinate user needs and wider aspects of synchrotron research among the three countries.

Diamond, which would cost £140 million (US\$237 million) over five years, would replace the existing Synchrotron Radiation Source (SRS) at Daresbury in Cheshire. The Wellcome Trust has recently pledged £10 million to its costs (see *Nature* 398, 318; 1997). But a recent upgrade of the SRS has bought time for researchers, ensuring that the facility will remain "in good shape for another five years", says David Norman, director of the SRS.

The situation is more critical in France, where the government has "suspended" approval of Soleil, intended to replace the

Lure facilities (800 MeV and 1.85 GeV) in Paris at an estimated cost of FF1 billion (US\$174 million). Both current facilities are already outperformed by machines elsewhere, and are poorly geared to meet the need for bright sources of soft X-rays and ultraviolet radiation spectra by biology applications, the area of synchrotron use where demand is greatest.

The French government was about to approve Soleil when the process was interrupted by the snap general election in June. Although the new government has increased spending on research, priority has been given to creating jobs. Funding for existing big science facilities has been cut, and the government is reluctant to make new large commitments.

Choosing a site for Soleil from among the many regions competing for the jobs and investment will inevitably cause discontent among the losers, say observers. They suggest that the government may choose to postpone the disappointment until after next year's regional elections.

Some researchers also claim the government has uncritically lumped Soleil with other 'big science' facilities. Dino Moras, a researcher at CNRS's Laboratory of Structural Biology in Strasbourg, argues that synchrotrons are "not big equipment used by a few, but a shared tool used by many".

Many at the meeting said that access to first-class national machines would be critical if Europe was to be competitive in many areas of biology, such as genome research. With both Britain and France each having user communities of around 2,500, the political response is out of touch with the "huge demand" in the scientific community, says one French scientist, who describes approving Soleil as an "absolute urgency".

Resentment at the lack of a French government decision is particularly strong, given that bidding regions have already agreed to meet half the costs from their own budgets, while CNRS and CEA have agreed to pay two-thirds and one-third of the remainder respectively. With the financing structure in place, the government's reluctance to give the project the green light "seems crazy", says Moras.

Meanwhile, staff at Lure are concerned that Soleil might not go to the Saclay site near Paris being proposed by the Paris region. If the facility were to go elsewhere, French synchrotron research would be set back by "years", says Roger Fourme, a researcher at the University of Paris-Sud at Orsay, and head of Lure's department of biology/biophysics.

Declan Butler

Korea holds firm to biotech expansion

[SEOUL] Despite a struggling economy battered by financial scandals, South Korea still plans almost to double its support for biotechnology next year as part of a programme under which government and industry have promised to invest US\$18 billion over a 14-year period. But Korean scientists still face obstacles before a strong biotechnology industry can be developed.

The Biotech 2000 programme was set up by the government in 1994 as an offshoot of the Highly Advanced National Project — otherwise known as the G-7 project — which aims to catch up with the world's seven leading industrialized countries in a broad spectrum of key technologies (see *Nature* **354**, 177; 1991).

Biotech 2000 aims specifically to catch up in biotechnology by the year 2007, the minister of science and technology, Sook-Il Kwun, said at the opening this month of a conference in Seoul on 'Bioscience Meets Engineering', co-organized by *Nature* and the Korea Research Institute of Bioscience and Biotechnology (KRIBB).

Almost US\$500 million has been invested by the government in the first four years of the programme, matched by about \$1 billion

from industry. Next year, the government will increase its budget by 80 per cent to \$312 million, while industry plans to boost its contribution by 30 per cent to \$470 million.

Seven ministries are participating in the project. Research focuses on strategic areas that include biomaterials, biomedical engineering, genome analysis, cell culture, breeding of biological resources, food and environmental biotechnology, bioenergy production and basic life science.

KRIBB in Daeduck science city in central Korea is a key player in the project, and funds for basic research are also dispersed to universities through the Korea Science and Engineering Foundation, including its centre-of-excellence programme which provides large amounts of funding to a few select laboratories (see *Nature* **364**, 383; 1993).

About 80 Korean companies are now involved in biotechnology, including large conglomerates such as Samsung and Lucky Goldstar that are more famous for their electronics products. Samsung, for example, has established a biomedical research institute at a new hospital it has built in Seoul, and plans to expand research facilities at the hospital in collaboration with Sung Kyun Kwan



Kwun: ambitious goals for biotechnology.

University, a private university in the outskirts of Seoul where Samsung recently funded the creation of a medical faculty.

But the government is also encouraging small and medium-sized companies to be involved in biotechnology. Some have traditional strengths in food and fermentation.

Korean scientists see big hurdles to overcome to bring biotechnology up to the level of Western countries. For example, although the government is deregulating the financial system to free up more venture capital, the capital available is small compared to the United States, Japan and Europe.

Also, venture capitalists in Korea tend to prefer to invest in areas such as computer software that provide a "quick cash return", Sunyoung Kim of Seoul National University told the conference. Kim has helped his postdoctoral fellows and graduate students to set up a venture business in a corner of his laboratory to develop retroviral vectors for gene therapy, and is turning to companies in neighbouring Asian countries to raise venture capital to compete with the "big boys" in the United States.

Another problem is the lack of a supportive culture in universities. Faculty members of Korea's top universities tend to spend their whole careers in the same department. This, combined with Confucian values that require respect for elders, makes it difficult for young faculty members to develop radical ideas and approaches.

"Living harmoniously is a much more important task [for faculty members] than painfully changing the system, even though that would be good for the university and society in general," says Kim. There is also strong resistance among many university researchers to involvement in business activities, which are considered outside the realm of academic research.

A shortage of people in universities and government with strong backgrounds in biotechnology also hinders development. But growing numbers of young Korean researchers are now returning from the West, introducing new approaches. This leaves Kim and others optimistic about the future of biotechnology in Korea, despite the many problems they face.

David Swinbanks

Troubled reactor wins five-year reprieve

[TOKYO] Japan's troubled prototype advanced thermal nuclear reactor, Fugen (pictured right), will continue operating for a further five years out of consideration for its 300 employees and because of its contribution to the local economy. The Science and Technology Agency (STA) has abandoned the idea of closing it down early.

The agency announced last week that Fugen, which comes under the Power Reactor and Nuclear Fuel Corporation (PNC), will continue operating until 2003, two years longer than initially planned. The decision came despite a series of accidents at the site, the most recent in October, when two plant workers were exposed to radiation during a routine inspection.

Fugen, in the reactor-studded Tsuruga City in Fukui Prefecture on the coast of the Sea of Japan, is the only heavy-water reactor among Japan's 50 operating nuclear reactors. The closure decision was made by the STA when a leak of tritium and heavy water in April was left unreported by the PNC for more than 30 hours (see *Nature* **386**, 746; 1997). Further investigations revealed that there had been 18 unreported cases of tritium leakage since 1992.

The incident followed a series of nuclear accidents and cover-ups by the PNC. Responding to growing criticism and public discontent about mishandling of nuclear



accidents, the STA formed a PNC reform committee to draw up measures for its reorganization.

After the committee's decision to shut the Monju experimental reactor and Fugen, the ruling Liberal Democratic Party's administrative reform council made plans to create a semi-private organization to take over part of PNC's core activities, including the operation of Fugen before closure.

The plan to extend Fugen's operations came after local authorities in Fukui, concerned about the local economy and employment, protested against the early closure. The reactor has been shut since the summer for inspection, and will restart some operations in December.

It is still not known when the new five-year term will begin. "It will start some time next year, but it could be as late as next October, when Fugen is handed over to the new controlling body," says one STA official.

Asako Saegusa

German minister nominated to head UN's green arm

[LONDON] Klaus Töpfer, Germany's minister for housing, has been nominated by Kofi Annan, the United Nations secretary general, as the next executive director of the UN Environment Programme (UNEP), based in Nairobi.

Töpfer's nomination, which was tipped at the beginning of last month (see *Nature* 389, 426; 1997), was expected to be ratified by the UN General Assembly in New York this week. A former German environment minister, he will replace the Canadian Elizabeth Dowdeswell early next year.

Töpfer's appointment will be widely welcomed. UNEP has been in something of a crisis in recent years, in terms both of finances and loss of credibility with governments and environmentalists. "UNEP needs someone who can think big," says one observer. "All indications are that Töpfer will fulfil that role."

Seed company declines Monsanto's terms

[LONDON] The US seed group Pioneer Hi-Bred has refused to incorporate a trait in its seeds that would make them resistant to a brand of herbicide manufactured by Monsanto. Charles Johnson, Pioneer Hi-Bred's chief executive, said in a letter to the company's customers that Monsanto's terms and conditions — which include passing on a technology fee to customers — would encourage a monopoly position for Monsanto.

"We believe that corn growers do not want a single company in a position to control a large number of new traits or combinations of traits," he wrote. But Monsanto has dismissed the company's complaints. It says that Pioneer Hi-Bred, whose sales account for nearly half of US seed corn, was taking a negotiating stance, and that most of its customers have accepted the concept of technology fees.

India announces brain research centre

[NEW DELHI] India's Department of Biotechnology (DBT) has announced that it is setting up a National Brain Research Centre (NBRC) in New Delhi "to enable Indian scientists catch up in the front line areas of basic research on the brain". The DBT will spend about US\$10 million over the next five years on this project, which is expected to be operational from March 1998.

The NBRC will fund and coordinate research on the brain in five existing medical institutions. It will promote research in developmental neurobiology, neuro-

immunology, neurogenetics, ageing, neuronal modelling and artificial intelligence, and drug abuse. Manju Sharma, DBT's secretary, says that brain research "has been identified as an area of high priority for basic interdisciplinary research".

Record ozone depletion in Arctic this year

[LONDON] Scientists observed record ozone depletion in the Arctic stratosphere during March of this year, with levels recovering in April. Writing in the latest issue of *Geophysical Research Letters* (24, 2689–2692; 1997), Paul Newman and colleagues from the NASA Goddard Space Flight Center in Greenbelt, Maryland, say that values of ozone in the Arctic during March were 21 per cent lower than usual.

Ozone values in a small region near the pole were approximately 40 per cent lower than normal. The researchers attribute the loss of ozone to the continuing effects of chlorine gases. The reactions between the chlorine and the ozone were activated by low temperatures, weak levels of sunlight, and high levels of total inorganic chlorine.

Iran ratifies convention on chemical weapons

[LONDON] Iran has ratified the United Nations chemical weapons convention, bringing the number of ratifications to 104. It follows recent ratifications from India, Jordan, Pakistan and Russia. Iran must now submit within 30 days of ratification a list of facilities involved in the production or storage of chemical weapons. Inspectors from the Organization for the Prohibition of Chemical Weapons based in The Hague will then be dispatched to verify Iran's inventory.

Germany eases access for foreign students

[MUNICH] Legal barriers to foreign students who wish to study in Germany are to be reduced. Following complaints from German science officials about unnecessary restrictions (see *Nature* 389, 323; 1997), Manfred Kanther, the interior minister, and Carl-Dieter Spranger, the minister for economic cooperation and development, announced earlier this month that agreement had been reached on looser rules for foreign students.

In future, for example, overseas students will merely have to prove that they have sufficient funds for one year — not for the entire period of their studies in Germany — and they will no longer be required to demonstrate proof of accommodation before entering the country. Since 1995, Spranger has been keen to encourage student exchanges with India, Brazil and Indonesia.

But German legislation has in many cases put young people off coming to Germany for a university education.

'Queen's Lectures' revived in Berlin

[BERLIN] The Technical University of Berlin, set up in 1946 by British occupying forces, has revived its 'Queen's Lectures', established by Queen Elizabeth II on a visit in May 1965. The lectures were originally given in Berlin by a prominent British scientist on each anniversary of the visit, but the tradition was dropped in 1975.

On the university's fiftieth anniversary, Rosemary Spencer, the British ambassador in Berlin, announced the resumption of the lectures. The first in the new series will be given on 1 December by John Krebs, chief executive of the Natural Environment Research Council, on the subject of 'Science and Sustainability'.

'Cannabinoid medicines should be legalized'

[LONDON] The British Medical Association (BMA) has proposed that medicines derived from cannabis be made legal to help treat disorders such as cancer, and to make easier their use in research. Cannabis and its derivatives are claimed to be effective in the treatment of nausea caused by cancer chemotherapy, pain, anorexia and epilepsy.

The BMA report, *Therapeutic Uses of Cannabis*, recommends that researchers in the United Kingdom should be allowed to use certain cannabis-derived compounds without needing a licence under the Misuse of Drugs Act. It also says that the World Health Organization should advise the United Nations Commission on Narcotic Drugs to remove certain cannabinoids from the UN convention on psychotropic substances.

Russia and Britain link defence research

[LONDON] General Igor Sergeev, the Russian defence minister, and his British counterpart, George Robertson, have signed an agreement in Moscow creating a commission on cooperation between their two ministries which will recommend areas for joint scientific research and the exchange of information on the procurement of equipment.

The chief scientist of the UK Ministry of Defence is expected to visit Moscow next year. The UK Defence Evaluation and Research Agency, which already has an office in Moscow, is expected to play a key role in strengthening scientific links between the two countries. The agency was reported this month to be the subject of a new review by the British government on whether it should be sold off to the private sector.

Kyoto meeting will seek to build bridge over troubled water

Stormy negotiations are expected during the third meeting of parties to the UN Framework Convention on Climate Change, which opens in Kyoto in ten days time. But optimists hope that significant agreement can still be reached.



When delegates from around the world gather in Kyoto next month, they will bring sharply clashing perspectives on the action they consider is required to limit greenhouse gas emissions.

As these positions collide at the summit, many will be tempted to see the inevitable tensions and disagreements as stemming largely from a single factor: the conflict between US intransigence and the interests of the rest of the planet.

With Europe supporting 15 per cent cuts in emissions by developed countries, and accepting that developing countries can stay outside the agreement until the rich countries have acted, the US proposal that emissions should be stabilized at 1990 levels in exchange for new commitments from the poor countries will attract scant support.

Some appear to dismiss the US position as merely a mixture of greed and short-sightedness. Robin Cook, for example, the British foreign secretary, has attributed some of it to the fact that one-third of US congressmen have no passport.

But closer analysis suggests that, as in any diplomatic negotiations, all countries will arrive at Kyoto with positions that substantially reflect their self-interest.

"It's a hard problem for the United States because the benefits are significant," says Jonathan Wiener, an economist at Duke University in North Carolina and former official at the White House Office of Science and Technology Policy. "But the costs are also significant."

The United States has a vocal scientific community and a large population of liberal-minded graduates who would no doubt look favourably on any administration that acted on global warming. But it also has emissions well in excess of any likely target — and faces significant costs in reducing them.

The positions of other countries can be assessed in a similar cost-benefit light. Political leaders in Germany and the United Kingdom stand to gain politically from backing an agreement to achieve steep reductions in emissions — and face relatively small costs in achieving such cuts, since they have already reduced domestic emissions since 1990, the benchmark year from which any agreement must be set.

For Germany, this happened because it absorbed the old East Germany in 1990, and has since closed down much of its heavily-polluting industry (Russia and the rest of Eastern Europe has shared in this emissions 'windfall'). In the United Kingdom, Conser-

vative Prime Minister Margaret Thatcher succeeded after a bitter battle with the nation's miners in closing much of the coal industry and starting a 'dash for gas' which reduced British emissions just after 1990.

Cost-benefit analysis

The statements of Tony Blair and Helmut Kohl must also be seen in the context of the fact that carbon emissions in both Germany and the United Kingdom have recently begun to rise again, increasing by 2.1 per cent in Germany last year, and 2.9 per cent in Britain during the same period.

US Vice-President Al Gore pointed out during a climate change conference last month at Washington's Georgetown University that it was relatively easy for Europe to achieve the goals it is suggesting (see *Nature* 379, 531; 1997). "It's kind of a freebie for them," said Gore. If Kyoto were a high diving competition, he suggested, "they'd not get a very high 'degree of difficulty' score for their efforts."

Cost-benefit analysis also comes into play in the case of countries which advocate inaction — namely Australia and the oil-producing countries of the Middle East — as these face high compliance costs, and would derive minimal domestic political benefit from tough international action.

And for the poor countries, although the costs of reducing their emissions are relatively low, so too are the short-term benefits. The governments of these countries often have more immediate priorities — such as feeding and schooling their populations, and building an industrial base — which tend to outweigh environmental concerns. They also signed an agreement in Berlin in 1995 under which the rich countries promised to act first.

When President Jiang Zemin of China was harangued by Clinton and Gore in Washington last month over what China was going to do about global warming, he is said to have responded with an assured silence. Many in the developing world would applaud that, still convinced that the problem is not of their making.

So with Europe making what appear to be relatively easy commitments, Australia and the oil-producing Arab states trying to undermine any treaty, and the poor countries largely waiting on the sidelines, many feel that the outcome of the Kyoto meeting will depend primarily on the United States.

The US position has emerged in the usual Clintonian style — at the last minute, after a protracted and public battle between advisers of different hues. As Madeline Albright, the secretary of state, said at Georgetown, "more so than any other subject, this involves every single department of the US government. It is a huge challenge to all of us."

In retrospect, it was a challenge that the

CO ₂ emissions from fossil fuel combustion (10 ⁹ tonnes of carbon)		
	1996	% change 1990-96
North America	1.75	+8.2
Latin America	0.33	+13.2
EU 15	0.96	+0.8
CIS, C, E Europe	0.90	-31.0
Middle East	0.25	+41.0
Africa	0.22	+19.0
Asia/Pacific	2.00	+31.0
Total OECD	3.27	+7.8
Developing countries	2.34	+32.0
World	6.51	+6.4

Source: World Energy Council

administration failed to meet. The Berlin mandate required parties to state their position by June of this year, but the United States waited until 22 October. The delay was meant to protect the position from attack and strengthen America's hand in negotiations — but it has done neither.

One effect of the delay has been to allow opponents of any deal being reached in Kyoto to set the agenda. Opponents have spent 1997 hammering the treaty as unfair, because it required the United States to act, but not China, India or Mexico.

Clinton on the fence

The complaint was taken up on Capitol Hill by centrist Democrats, including Senator Robert Byrd (Democrat, West Virginia), who used it to undermine potential support for a treaty. Republicans — who fear being identified as friends of the polluters — have been able to stay quiet on the issue, while supporting a resolution from Byrd advising the administration not to sign any treaty unless the poor countries agree unspecified action to limit emissions, on the same timescale as the rich countries. The Senate backed the resolution unanimously.

Meanwhile, the administration has itself

been split. Liberals such as Bruce Babbitt, the interior secretary, have chosen to confront treaty opponents, stating that energy companies use “a couple of pet scientists — who happen to be on [the companies'] payroll” to attack climate science. Babbitt apologized to a meeting of the Union of Concerned Scientists in September for being “shrill” in his attack — but added: “they deserve it”.

But Clinton has remained firmly on the fence. At the Georgetown meeting, he characterized the argument as “people of goodwill, bringing forward many honest disagreements”. That drew wry smiles from scientists, environmentalists and industrial lobbyists, who have been engaged in a bitter public dogfight on the issue for several years.

The Clinton plan features a multi-point proposal to begin voluntary reductions in US emissions. It calls for unspecified “developing country participation” in any treaty, but its most significant feature is perhaps the pledge to start a market-based emissions trading system, “both domestically and internationally” in 2008, “after a decade of experience” with the voluntary measures.

Developing country participation, a ‘joint implementation’ scheme and emissions trading are locked together in a com-

plex triangular relationship (see page 219) which holds the key to any deal that may be struck in Kyoto.

The Clinton position may be low on specifics, but it remains a finely-tuned compromise between competing US interests. Unfortunately for those hoping for stronger action at Kyoto, any significant shift will knock that compromise off balance in a way that Clinton seems unprepared to risk.

Most notably, any move to reduce emissions from developed countries to below their 1990 levels will unleash a barrage of fire from those keen to derail the treaty. Clinton will be able legitimately to plead that no such reduction would be ratified by the US Senate. Conversely, any move to bring a significant number of developing countries into the agreement, in defiance of the Berlin mandate, will be an uphill task.

This month there are signs that some of the more advanced developing countries might be prepared to take on voluntary commitments in exchange for a per-capita based system of calculating emissions targets. But it remains hard for many to see what incentive will persuade the poorest countries to sign the kind of agreement Senator Byrd has in mind.

Colin Macilwain



Equity is the key criterion for developing nations

If the Kyoto meeting fails to reach a satisfactory outcome many leaders of developing countries are aware that they may be asked to share responsibility for the failure. The question occupying the minds of top civil servants from Brasilia to Beijing, already under pressure to reduce their greenhouse gas emissions sooner than they want, is simple: how should they respond?

So far, the Group of 77 developing countries, which includes India, African states, the Middle East, Southeast Asia and the whole of Latin America apart from Argentina, has been united in its opposition to the US proposals (see above) for developing country commitments.

Unsurprisingly, the poorer countries argue that the rich must take the lion's share

of responsibility for combating global warming, on the grounds that they are the prime cause of current problems. Until this happens, the G77 countries say they will also refuse on principle to discuss the issue of emissions trading or joint implementation.

But the G77 is an uneasy coalition. At one end of its spectrum of opinion are the oil states, including Saudi Arabia, Kuwait and Venezuela, which would prefer a weak agreement that does not harm sales of oil. At the other are the small island states and low-lying countries, such as Bangladesh, for which a weak agreement may spell environmental catastrophe. In the middle lie India, China and the other large industrializing countries of the Far East and Latin America, who will resist any agreement that would harm their industrial growth.

How developing countries choose to respond to the US proposals remains the key question up to, and even after, Kyoto. There are three possible scenarios. Under the first, the US conditions will be unanimously opposed, even if this means no agreement at Kyoto. Under the second scenario, developing countries will split between those who agree to support the United States, and those who refuse.

The third scenario would see developing countries as a group striking a deal with the United States in which they agree to reduce their emissions at some point in the future, but with the United States providing them with something in return.

Despite their public opposition to the

idea of immediate commitments from developing countries, many officials from the G77 and China (which is not a member of the group) seem reconciled in private to the idea of a 'non-binding' side agreement — a 'Kyoto mandate' — attached to the main treaty. Under this, developing countries would make a non-binding promise to reduce emissions by a certain amount from a specified date. In return, the United States is likely to be asked not to block agreement on targets that would enable developing countries to reduce emissions to a per-capita limit — instead of a flat percentage reduction.

The countries that support this stance believe it to be a more equitable way of distributing emissions reductions.

A per capita-based solution would set an emissions limit, or 'cap', to a specified number of tonnes of carbon per person a year. Countries emitting more than this would agree to reduce their emissions to the required cap by an agreed date. Countries that emit below the cap would be allowed to increase their emissions up to the limit (see graph right).

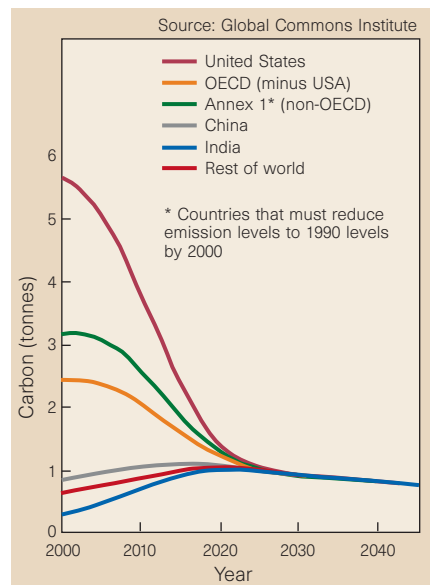
Per capita solution?

Those who have been promoting the idea that the world's emissions could converge on a single, per-capita figure include the London-based environmentalist lobby group, Global Commons Institute. Aubrey Meyer, GCI's director, says that, if a per capita strategy were to be followed, global concentrations of carbon dioxide could stabilize by 2030 at a level of 450 parts per million by volume of the atmosphere. (This is still well above the pre-industrial level of 280 parts per million; atmospheric carbon dioxide concentrations at present are 360 parts per million.)

Partly at Meyer's suggestion, this idea has already been formally adopted by the African group of countries, led by Zimbabwe. A variation of this strategy also lies behind the decision by European Union member states to back an average 15 per cent reduction; countries such as Portugal and Greece would be allowed to raise emissions, while others, such as the United Kingdom and Germany, would reduce theirs by more than 15 per cent.

But some European countries — the United Kingdom in particular — remain nervous about the idea of differentiated responsibilities based on per capita emissions being applied elsewhere. According to a senior British official, this is primarily because of the difficulty in deciding the level at which the cap is set.

A per capita solution is also opposed on strategic grounds by most environmentalist groups, in particular the Climate Action Network, an umbrella group of most of the world's climate-related non-governmental organizations. Indeed, CAN is lobbying developing countries not even to respond to the United States' proposals.



How per capita emissions of CO₂ from fossil fuel sources could converge to 1 tonne per capita (450 parts per million by volume) by 2030.

Jennifer Morgan of CAN in the United States says the organization sees the main problem as the timing — not the principle — of a proposal on per capita emissions. CAN will oppose anything that reopens the original terms of the climate convention in which developing countries are exempt from reducing their emissions.

Morgan describes the introduction of the developing country issue at Kyoto as a flawed strategy which could imperil the prospect of a legally binding treaty. She fears that the United States might use the developing countries as an excuse to veto the protocol if its terms are not to its liking.

Heavyweight support?

But a per capita based solution has found enthusiastic supporters in the European Parliament, as well as in the Globe network, an organization comprising parliamentarians with an interest in environmental issues.

Globe is engaged in its own lobbying campaign. When governments and environmentalist groups were protesting against the Byrd resolution in the US Senate, Globe took what some saw as the extraordinary step of lobbying senators to support it, arguing that the resolution is a route to procuring agreement at Kyoto by getting the United States to agree to per capita emissions in exchange for developing country reductions.

If it is to go further, however, the per-capita idea needs the support of heavyweights such as China and India. China is known to be sympathetic, and said so at a recent conference in Beijing. India is believed to hold a similar view, but continues to maintain an unsettling silence.

China's position reflects a debate between traditional Communists, who strongly oppose the US line partly on ideological

grounds, and a more pragmatic breed of politician ready to engage with the United States if a long-term benefit for China can be found. On climate change at least, the latter group seems to be winning the argument.

In a speech last month in Beijing, Song Jian, president of China's Council for International Cooperation and Development, said: "China bears no responsibility for reducing greenhouse gas emissions." But he added: "When we ask the opinion of people from all circles, many, in particular scientists, think that the emission control standard should be formulated on a per capita basis." Sir Crispin Tickell, warden of Green College, Oxford, a member of this council, was present at Song's speech. He says this is the clearest indication likely to be given of China's preferred route to emissions reductions.

India, on the other hand, has maintained an uncharacteristic silence about greenhouse gases since the change of government last year which saw the departure of the activist environment minister, Kamal Nath. This could be because India is unwilling to engage with the United States until Kyoto. Anil Agarwal, director of the Centre for Science and Environment in New Delhi, has a simpler explanation: India's climate policy, he says, is in total disarray.

But Kilaparti Ramakrishna, director of the science and public affairs programme at the Woods Hole Research Centre in Massachusetts, says that India may yet emerge as a major player. "Responsibility for climate policy has been given a higher political priority," he says. "It used to fall under the remit of the Department of Environment and Forests. But recently it has been taken over by the more powerful Foreign Office, which thinks more in terms of north-south [global] equity. That is a significant development," he says.

The United States, meanwhile, has neither ruled in or out the question of per capita greenhouse cuts. But most US administration officials remain unconvinced about the idea. There is the obvious concern that, under this strategy, the United States would have to make the largest reductions. There is also the view in some quarters that it seems to reward countries — such as China — with large populations and relatively low energy consumption.

Finally, the idea of an equity-based distribution of responsibility to reduce global warming strikes some as being ideologically tainted. In the words of one US official, "To me this is global Communism. I thought we'd won the Cold War."

But the idea still has its strong supporters. Indeed, many now feel that an international commitment to per capita based targets, rather than absolute goals, is most likely to produce a solution at Kyoto that both rich and poor countries will be prepared to swallow.

Ehsan Masood

Waning influence provokes green groups' frustration

The prospect of a global treaty on greenhouse gas emissions represents — at least in principle — a hard-won victory for the environmentalist movement. Indeed, for groups such as Greenpeace and the World Wide Fund for Nature, Kyoto should be pay day after more than a decade of campaigning. Why, then, is the mood in the green corner one of despair, frustration and anger?

Much is due to the fact that the treaty in sight is much weaker than the one they have been fighting for. Although such groups are sufficiently experienced to know that the final outcome of international negotiations will always fall short of their demands, they are angry that the likely result at Kyoto will be even less than their worst expectations, says Tessa Robertson of Greenpeace International.

One reason, according to some environmentalists, is that Kyoto is unlike several previous meetings on environmental issues — including the 1992 Earth Summit in Rio, at which the most governments had to do was to put their names to a set of vague promises on protecting the world's climate. A second reason is that the environmentalist movement is divided. These divisions have made more difficult the task of influencing key governments in the negotiations.

In general, the political power of environmentalist groups in international negotiations rests on their ability to influence pivotal governments from both developed and developing countries. A close understanding is critical to their efforts to shape the outcome of such negotiations, in which they are officially only observers, not participants.

During meetings such as Kyoto, 'green' activists regularly exchange information in private with friendly governments, and pass on selected — and measured — snippets of information to those who they can trust or, such as journalists, help to spread their message. Friendly governments are provided with useful items of gossip; less friendly governments are aggressively lobbied during lunch-breaks and in the corridors.

"Information is our greatest asset. It is our greatest source of strength. It is where our power lies," says Delia Villagrasa, director of Climate Network Europe in Brussels. "We don't have the money, but we do have access to the main players."

At Rio, environmentalists were close to countries such as Canada and India, both of which assumed strong and influential leadership positions in discussions on the climate convention. But changes in government in both countries mean that neither can be relied upon to support the environmentalist cause.

Not all has been lost. Canada's role has, to some extent, been replaced by the more powerful European Union, and in particular by Britain, whose greenhouse target is one of the toughest on the table, and closest to the environmentalists' demand of a 20 per cent reduction in emissions by 2005.

But India's role has been harder to replace. Indeed, its loss means that the greens now lack an ally among the 'influential developing countries'; the long-standing support of the 42-member Alliance of Small Island States is not enough. The environmentalists need a big hitter.

One priority or many?

An African country might have played this part. But a split in the environmentalist movement has meant that Africa's loyalties have been pledged elsewhere to another environmentalist group. This may sound like routine activist infighting, but it has important implications for the outcome at Kyoto. And it cuts to the heart of the debate about the nature of environmentalist activism.

Environmentalist groups fall into two broad categories. There are those, such as Greenpeace and other members of the broad Climate Action Network (CAN), for whom protecting the environment will always remain a priority above all else. They argue

that human survival demands deep cuts in emissions — and that nothing less will do.

But there are others, particularly in developing countries, for whom environmental protection is part of a wider agenda to reduce poverty and increase 'quality of life'. "The old debate of environment versus development is irrelevant," says Aban Marker Kabraji, regional director for south and southeast Asia at the International Union for the Conservation of Nature. "Issues of poverty alleviation, governance, and the economy are as important as the latest statistics on sea level rise."

Integrating environment with development is an attractive way of 'selling' environmental awareness to developing countries. It was sufficient to convince Rungano Karimanzira, leader of Zimbabwe's delegation to Kyoto, and deputy director of the country's Department of Meteorological Services, not to support CAN on the question of per capita emissions (see page 217). As Zimbabwe happens to head the group of African countries, the rest of the continent took little persuading to follow suit.

For Anil Agarwal, director of the Centre for Science and Environment in New Delhi, CAN's failure to influence more governments boils down to its unwillingness to see climate change in a broader context. "[Environmentalist] non-governmentalist organizations lost the plot after Rio," says Agarwal. He says they restricted themselves to campaigning for reduced emissions when they should have worked out detailed positions on related issues, and then tried to sell their ideas to governments. **E.M.**



Joint actions may hold the vital formula

Is the United States ready to cut its carbon emissions, as the rest of the world demands? Not a chance. Are the poor countries ready to promise to cap their own emissions, as the US Senate is demanding? No way. Is the Kyoto meeting therefore heading for abject failure? Not necessarily.

The differences between the two sides could be accommodated. And experts agree that the essence of any such accommodation will be the practices known as 'joint implementation' and emissions trading.

'Joint implementation' is the term used by economists to describe projects carried out by the rich countries in poor countries in order to help meet the former's targets for reducing greenhouse gas emissions.

Instead of slapping five cents on the price of a gallon of gasoline, for example, the United States could attempt to meet its own reduction targets by helping to construct a hydro-electric scheme in Guatemala, alleviating the need for that country to generate electricity by burning fossil fuel.

Under the related concept of emissions trading, governments — or even corporations — could buy and sell emissions credits. If Japan could not meet its own emissions goal, for example, it would be able to buy 'credits' from a nation such as Russia that was on course to meet its target.

Economists believe that such a market would redistribute emission cuts to wherever costs are lowest. Indeed, trading in emis-

sions of sulphur dioxide has been tried out between corporations in the United States, and has been highly successful in reducing the costs of cutting pollution.

But vast obstacles remain to the use of either joint implementation or emissions trading in a global scheme to cut greenhouse gas emissions. In the case of joint implementation, a programme of pilot schemes introduced as part of the 1995 Berlin mandate has so far achieved little.

Europe and the poor countries want the pilot programme to continue for a couple of years on its current basis. But the United States believes that the scheme has been slow to take off because it does not give participants any 'credits' against their own emissions targets — and wants it reformed to do precisely that.

Joint implementation need not only give credits to the rich, donor country, but could do so to the poor, recipient nation as well. But many poor countries don't want that, as it would imply their acceptance of the need for caps on their own emissions. "They see joint implementation as the start of a slippery slope" towards accepting caps, says one diplomat.

According to Bob Dickson, head of the US government's joint implementation office, "We'd like to end the pilot phase and get an agreement for joint implementation with crediting to all parties." But after two years of trying, the United States has only

won the support of Costa Rica, and possibly Argentina, for its vision of joint implementation.

Emissions trading is, if anything, even more fraught. A trading regime would probably be limited at first to those rich countries — the United States, western Europe and Japan — that have the legal and technical equipment to monitor emissions and implement a trading scheme.

Trading could occur at two levels: between nations, and between corporations within each nation. The latter would be relatively straightforward in countries with competitive, private-sector energy markets, such as the United States.

But any global scheme would be far more complex, as it would require global policing. Commentators such as Ed Parson, a specialist in emissions trading at Harvard University's John F. Kennedy School of Government, point out that sulphur dioxide trading only works in the United States because the powerful Environmental Protection Agency is there to enforce it.

Some critics also ask who would prevent governments from interfering with the market to defend their regional and national interests.

Trading would "require governments to relinquish a huge amount of power," says Parson. "International trading is the way to go — but it is naive to think that we have worked out how to do it."

C.M.

What to look for at Kyoto

The United Nations Framework Convention on Climate Change opened for signature at the 1992 Earth Summit in Rio de Janeiro, at which countries agreed to reduce their emissions to 1990 levels by 2000 (a goal that now appears highly unlikely to be achieved). The convention was strengthened three years later at a meeting in Berlin at which signatories agreed not to require new commitments from developing countries. At Kyoto, the plan is to go further and set legally binding targets for the period beyond 2000.

● The Alliance of Small Island States

The oldest, most ambitious and — politically — least realistic target is that proposed by the 42-member Alliance of Small Island States. With the most to lose from rising sea levels, member countries want strong action, and fast. This has translated into demands for a 20 per cent reduction in greenhouse gas emissions from 1990 levels by 2005.

● The European Union

The 15 member states of the European Union have agreed to propose a 15 per cent

reduction from 1990 levels by 2010 for all developed countries. The EU has also proposed an interim target of a minimum 7.5 per cent reduction in emissions by 2005. Reductions will be made to a 'basket' of three gases: carbon dioxide, methane and nitrous oxide.

The ingenuity of the EU proposal is its 'bubble' approach, averaging out member states' emissions. This would allow poorer member states to increase their emissions as richer states cut down more than the average. It also accommodates France, which is keen only to stabilize its emissions at 1990 levels on the grounds that they are already relatively low because it relies largely on nuclear power rather than fossil fuels.

● The United States

The United States will go to Kyoto with one of the weakest proposals on emissions targets — a promise merely to stabilize at 1990 levels by between 2008 and 2012, with a further unspecified cut by 2017. But administration officials point out that even stabilization at 1990 levels would amount to a 15 per cent cut



for the United States from present levels — and that emissions are continuing to rise.

In addition, US officials want 'meaningful' participation from major developing countries, including a willingness to participate in emissions trading and joint implementation. Observers believe that the United States may possibly relax its stance on targets slightly in the closing stages of the meeting if some of its other conditions are met.

● Japan

Japan has taken much flak for its proposal to reduce emissions by 5 per cent from between 2008 and 2012 for a three-gas basket of carbon dioxide, methane and nitrous oxide. But this proposal could well emerge as the only realistic outcome at Kyoto. If so, Japan's

much-maligned 'softly, softly' consensual approach to the negotiations will have been vindicated, and its first foray as host of a major international agreement deemed a success.

As the host of the meeting, Japan has been pilloried by some for not showing more leadership. But Japanese officials are in close and constant touch with Washington and major European and G77 capitals. It is therefore little surprise that their proposal neatly falls between those backed by the first two, while incorporating some of the demands of the G77.

Japan's proposal includes elements of other demands, such as lower targets for countries with low population growth and low incomes per head. The proposal also

calls on 'advanced' developing countries to assume voluntary commitments.

● Developing countries

The Group of 77 developing countries tried to scoop the US proposal last month by tabling their own proposal hours before Clinton's announcement. The G77 proposal is a slightly modified version of that from the European Union: it suggests that targets be set for individual gases, not a basket of three, and achieved domestically, without emissions trading or joint implementation. The group also wants a 35 per cent reduction in emissions by 2020.

The G77 proposal includes a demand for a compensation fund for 'economic impacts' of climate change policies. This demand spans a variety of interests, from those of the oil-producing states, which want to be compensated for revenues from lost oil sales, to those of the small island states, which want compensation if they suffer because of delays in international action.

● Brazil

One of the most sophisticated proposals comes from Brazil. Largely the work of Gylvan Meira, head of the Brazilian Space Agency, it suggests that targets be based on historical emissions; in other words, that those countries that began to emit carbon dioxide from the beginning of the Industrial Revolution should reduce the most, and vice versa. It also includes penalties for countries which overshoot their targets, to be paid into a fund to finance clean technology projects in the developing world.

● Australia

Australia is one of the few countries to have publicly declared its opposition to legally binding targets, despite appearing to be brought into line last month at the Commonwealth Heads of Government meeting (see *Nature* 389, 893; 1997). Australia's industry is dominated by companies that

Obstacles to an agreement

Success at Kyoto will need agreement on a method of calculating emissions reductions. The United States favours what has become known as the 'net' approach. According to this method, a country's inventory of 'man-made' carbon emissions will include carbon released into the atmosphere from the clearing of forests. It will also take account of carbon removed from the atmosphere from, for example, the planting of trees. This is controversial, partly because it is not clear how this carbon will be calculated.

Another issue will be the 'basket' versus the 'gas-by-gas' method of calculating emissions. The European Union supports the idea that reductions should be made collectively to a basket of three gases: carbon dioxide, methane and nitrous oxide. But environmentalist groups oppose this on the grounds that it would allow countries to make disproportionate reductions to methane and nitrous oxide, in comparison to carbon dioxide, which constitutes 80 per cent of developed country greenhouse gas emissions.

Also at issue will be the period over which reductions can be made. Some countries — the United States again — would like five years to reduce emissions. Others want specific annual targets.

Finally there is the crucial question of which 'legal instrument' to use. If countries do agree on targets, the climate convention will need to be changed. The change could take the form of an amendment to the convention. This will require signatures from at least three-quarters — more than 120 — of the countries which have signed and ratified the convention. But the amendment will not enter into force — the point at which emissions targets become legally binding — until all these countries have ratified the change to the convention in their national parliaments. This is not

expected to happen before 2010.

The alternative method of changing the convention is through what is known as a 'protocol'. The main advantage of a protocol over an amendment is that countries have more freedom to decide when it enters into force. They can, for example, decide entry into force after it has been ratified in the national parliaments of, for example, just 50 countries. But a protocol's drawback is that it needs a consensus of all the parties. In other words, a single, dissenting country can veto the whole process.

European government lawyers anticipated this potential difficulty nearly a year ago, and have tabled an advance amendment to the convention which says that a protocol should be allowed to be adopted at Kyoto by a three-quarters majority vote. This is a high-risk strategy and bound to be opposed by countries such as Saudi Arabia and Australia. "When you've got 48 hours to go [at Kyoto], everyone's dead tired, and one country is blocking progress, the chairman will need to pull a rabbit out of the bag," says one government



BRIDGEMAN ART LIBRARY/HOKUSAI, 'THE GREAT WAVE OF KANAGAWA'

Support for genetic diversity project

Sir—I am writing in response to a recent article on a report from the US National Research Council, *Evaluating Human Genetic Diversity* (*Nature* **389**, 774; 1997). I chaired the committee that wrote the report.

The article was correct in stating that the committee does not think that the “consensus document” drafted in 1993 and which has been construed as the Human Genetic Diversity Project sets forth a clearly articulated, sharply defined proposal that the committee could evaluate. However, this does not constitute disapproval of the concept behind the proposal; indeed, the committee strongly endorsed the scientific merits of a global study of human genetic variation. A global study performed in a way that would not reveal the identities of the DNA donors or compromise their rights could be of tremendous value for researchers who study human origins or anthropology. The committee believes that such a survey, if performed to protect the rights of individual donors, does merit federal funding.

And your article is in error when it asserts that the committee recommends that federal funding agencies “should confine support of human genome diversity work to projects inside the United States”. Our recommendation reads: “These agencies should focus their financial support, at least initially, on projects originating in the United States and expand their support to the international scene only after the US activities are successfully launched.” The word ‘originating’ is not synonymous with ‘inside’ nor did we expect it to be so construed. Our intention was to ensure that research, wherever it might be pursued geographically, would have to meet all of the ethical and legal restrictions at present placed on human genetic research funded by federal agencies in the United

States. We believed this aim could be achieved only through restricting research applications to those that sought US federal monies.

William J. Schull

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Sir—The News item by Colin Macilwain (*Nature* **389**, 774; 1997) covering the recent prepublication report of the US National Research Council (NRC) about research on human genetic variation as proposed in the Human Genome Diversity Project (HGDP), cites only a few of the findings and recommendations in a comprehensive, informative and, by our reading, favourable evaluation prepared by the expert NRC reviewing committee. The News item reflects your journal’s apparent continuing bias against this scientifically acclaimed project.

The task of the NRC committee, formed at the request of the US National Science Foundation (NSF) and National Institutes of Health (NIH), was to assess the scientific value, technical aspects and organizational requirements of a systematic worldwide survey of human genetic variability, as well as the ethical, legal and social issues raised by the project.

The report, some 81 typewritten pages, contains, in addition to an executive summary and an introduction and background, five chapters dealing with, respectively, scientific and medical value of the proposed research, population sampling issues, sample collection and data management, ethical and human rights issues with respect to the project, and organization and funding support aspects.

The reviewing committee has concluded that “a global assessment of the extent of

human genetic variability has substantial merit and warrants support” and noted that the “most well-developed and widely recognized proposal for conducting such a survey is known as the... HGDP”.

Contrast the committee evaluation with your headline, “Diversity project ‘does not merit federal funding’”, echoed in the first paragraph of the News item, a phrase that is not found in the NRC report.

The NRC committee indeed suggests that the NSF and NIH should finance projects originating in the United States at least initially and “expand their support to the international scene only after the US activities are successfully launched”. These recommendations are understandably directed at US funding agencies and cannot and are not intended to refer to existing and future studies funded by agencies in other parts of the world.

Macilwain mixes roughly equal quantities of facts, in and out of date, with interpretations which do not reflect the NRC report in fashioning the News article. Projects that call themselves HGDP or reflect similar aims have started in half of the world.

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● The headline of the story was misleading. We reject the allegations of editorial bias. — Editor, *Nature*.

Patients and patents

Sir—Thomas *et al.* present data showing that the largest single category of patents published in 1995 that included claims for human DNA sequences was in the area of genetic diagnostics, and that 40 per cent of the patents they identified originated from US public-sector institutions (*Nature* **388**, 709; 1997).

We take exception to their conclusion that public-sector researchers realize that “patenting optimizes the chances of patients receiving benefits from their scientific research”.

The more likely explanation for this

observation is that researchers and their institutions realize that they can cash in on these discoveries from windfall profits from research supported in part by the taxpayers.

Rather than optimizing patient benefit, these patents threaten to curtail research, raise the price and lower the availability of testing, threaten patient privacy because of potential litigation and insert a troubling and unseemly profit motive into the dissemination and use of genetic testing services.

Thomas *et al.* also state that industry will not invest in treatments without adequate patent protection, but there is no reason to

believe that patenting of basic information about the human genome — particularly about naturally occurring human genomic sequences and the association of mutation with disease — is necessary to promote downstream therapeutic development. To our minds, the risks of these patents far outweigh the potential benefits, and they should be prohibited.

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Health effects of tropical smoke

Sir — Since August, large parts of Indonesia and Malaysia have been subjected to prolonged episodes of thick haze caused by smoke from intentionally set fires, chiefly in Sumatra and Borneo. As reported in *Nature*^{1,2} and on many Internet web sites³, the haze has caused a serious health emergency for millions of people and many species of animals and has adversely affected agriculture.

A report⁴ on the incidence of upper respiratory tract infections (URTI) in Sarawak, Malaysia, shows that the average daily hospital and clinic visits from 5 to 28 August numbered 593 compared with the average for 1996 of 346 visits per day.

A more recent plot of hospital and clinic visits in Sarawak⁵ shows a very significant increase in URTI admissions in September, exceeding 3,000 on 23 September. The incidence of URTI was associated with an extraordinarily high air pollution index (API) which exceeded 900 on 23 September. An API greater than 400 is considered life-threatening.

Although deforestation in Africa and South America has received considerably less notice than the Asian crisis, there was

considerable smoke over substantial portions of these continents during the 1997 burning season. Satellite observations show half of Brazil was covered by smoke during parts of August and September.

The consensus of a meeting of physicians and health officials in Alta Floresta, Brazil, on 27 August, which was organized to hear results of my continuing UV-B, optical depth and airborne bacteria studies there, was that half the local population was suffering from respiratory disease. Dr Antonio Sinkos stated that he saw "two to three patients per week [with respiratory disease] before the burning and now eight a day".

He and his colleagues expressed particular concern about the effects of the smoke on asthmatics, children and the elderly. Health statistics for Alta Floresta are now being compared with measurements of optical depth and UV-B.

Studies of the health effects of severe air pollution in urban areas are complicated by multiple pollutant sources. The importance of the data from Sarawak and Alta Floresta is that they provide important information about the health effects of a single source of pollution, biomass burning. When combined with measurements of sharply diminished UV-B associated with thick smoke, these data also provide an important opportunity to study the

increased incidence of disease caused by pathogenic bacteria and viruses which are ordinarily inactivated by solar ultraviolet⁶.

Although laws prohibit or regulate biomass burning in Indonesia and Brazil, enforcement is either lax or difficult. Until enforcement is achieved, there is a real possibility of smoke emergencies in future years. Countries at risk have a responsibility to prepare for future smoke emergencies, perhaps with the assistance of the international community and the UN Department of Humanitarian Affairs⁷.

Medical and scientific emergency response teams should be organized for immediate deployment to regions with serious smoke problems. Medical teams should be prepared to distribute suitable respirators (not merely dust masks), medical supplies and tracts that advise people why and how to avoid smoke. Scientific teams should be equipped with portable Sun photometers, radiometers and aerosol collectors. Observers in developing countries with limited budgets can assemble very inexpensive, accurate Sun photometers from readily available components^{8,9}.

A few dozen scientists in Brazil, the United States and elsewhere have considerable experience of making smoke-related measurements and could participate in an international response to

major smoke emergencies. All data should be freely shared among team members and scientists from the host countries.

The TOMS (Total Ozone Mapping Spectrometer) satellite instrument can reliably detect the presence of smoke over land¹⁰. Optical depth measurements of smoke in biomass burning regions can provide important calibration data for this instrument.

The close association of upper respiratory diseases with optical depth in Alta Floresta and the API in Sarawak suggest the possibility of using TOMS as an epidemiological tool to identify regions that might have smoke-related respiratory disease.

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Progress on bioethics blocked in Japan

Sir — Although I might agree with your opinion that “Japan’s bioethics debate lags behind thinking in the West” (*Nature* **389**, 661; 1997), it is misleading to say that “Japan’s cultural and religious background” or a “lack of understanding” account for this state of affairs.

What is really blocking progress in bioethics-related issues in Japan is, first, the inability of the medical profession to govern itself and, second, deficiencies in Japan’s approach to setting regulatory standards for research involving human subjects.

On the first point, any debate about bioethics is futile if medical practitioners and researchers are not subject to peer scrutiny and professional sanctions. In most countries, this is guaranteed by professional bodies with obligatory membership, similar to the General Medical Council in Britain or the German Ärztekammer. But there is no such organization in Japan.

The absence of a self-governing professional body and the subsequent lack of binding guidelines have created public distrust of the medical profession in Japan.

Although the Japan Obstetrics Society has issued guidelines to regulate reproductive technologies, these guidelines are regarded merely as ‘opinions’ and do not carry authority. This is the most noticeable difference between Japan and the West where bioethics is concerned.

On the second point, the principles governing human experimentation set out in the Nuremberg Code and the Declaration of Helsinki are generally regarded as the basis of contemporary medical ethics and bioethics, and national regulation should endorse these principles.

In Japan, only clinical trials of new drugs are subject to regulation based on these principles. Other medical procedures still at an experimental stage are often carried out as ‘treatment’ — not as clinical trials — and are therefore not subject to appropriate regulation. This situation is more problematical even than individual technologies such as organ transplants.

The task in Japan is organizational and political and has nothing to do with alleged Japanese cultural uniqueness or persisting public misunderstandings.

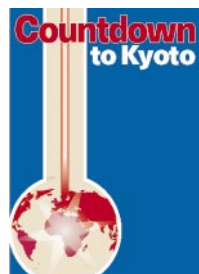
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Climate-change research after Kyoto

A scientific consensus that humans are influencing the climate will be behind any agreements on greenhouse-gas reductions next month. But how can climate research have an optimal influence on climate policy in the future?

Klaus Hasselmann



The issue of climate change, to be negotiated next month at the United Nations climate conference in Kyoto, Japan, is the most difficult environmental problem facing us. The impact of greenhouse-gas emissions will last for many centuries, far beyond normal economic and political planning horizons. As no effective way of disposing of carbon dioxide currently exists, most solutions to greenhouse warming call for a complete restructuring of our energy technologies, away from fossil fuels towards regenerative sources. This affects the very basis of industrialized economies, unlike the problems of stratospheric ozone, acid rain or urban smog. Further, the predictions of climate researchers are much less certain than policy-makers would like.

So how can climate scientists contribute to a solution? First, they can inform the public and policy-makers about their research, as done by the Intergovernmental Panel on Climate Change (IPCC)^{1,2}. Second, they should constantly strive to improve the reliability of their predictions. Third, they can study the complex link between pure research and policy-making^{3,4}.

As to the first of these, the 1990 IPCC report¹ compiled and reviewed by hundreds of experts worldwide, set the scientific stage for the first United Nations Conference on the Environment and Development in Rio de Janeiro in 1992, and for the follow-up Conference of the Parties of the Framework Convention on Climate Change in Berlin in 1995. Similarly, the IPCC Climate Change 1995 report² will provide the scientific basis for Kyoto agreements, and IPCC updates are expected for future negotiation rounds.

Improving predictions

It is now generally accepted that climate change through human activity is a reality. But model predictions of future greenhouse warming are still uncertain to within around 50 per cent, and the 1995 IPCC statement² that "the balance of evidence suggests a discernible human influence on climate" has caveats. How can prediction and detection of anthropogenic climate change be improved?

The physical laws underlying climate models of the coupled ocean-atmosphere system are basically well understood. Un-

certainities arise mainly through the coarse resolution of 200–300 kilometres imposed by computer limitations. Processes below this scale, such as cloud formation, or the sinking of cooled surface water at high latitudes that drives the deep ocean circulation, must be 'parametrized': that is, represented in terms of larger resolved scales. These limitations will be alleviated as more powerful supercomputers become available. But the computational demands will be great: doubling the spatial resolution of a global model requires halving the grid spacing in each of the three space dimensions and (for dynamical consistency) in the computational time step. This amounts to increasing the computational load 16 times. The benefits of improved resolution in a few years will be enormous, most pronounced on the regional scales near the resolution limit critical for assessment of climate change impact.

Analysis of data from satellites, international measurement campaigns such as the World Ocean Circulation Experiment, and global station networks has already led to improvements in physical parametrizations at subresolution scale, although more attention needs to be paid to incorporating into climate models better representations of the global biogeochemical cycles that determine the chemical constituents of the atmosphere.

Improved climate models would provide three clear benefits. First, they would allow more reliable predictions not only of changes in temperature and sea level, but also of other important climate properties that cannot yet be reliably computed, such as floods, droughts, hurricanes and mid-latitude storms. Many speculate that weather extremes have been increasing in both frequency and intensity, and that the increase is due to anthropogenic climate change. Although neither point has been clearly cor-

roborated, changes in extreme events are predicted for the stronger warming projected for the next century, including more intense El Niño events.

Second, improved models would lead to a better risk assessment of potential climate instabilities, such as the breakdown of today's global ocean circulation, the thawing of the large methane reservoirs in Siberian permafrost regions, or the break-up of the West Antarctic Ice Sheet.

Third, they would allow us to detect anthropogenic climate change with greater statistical confidence^{5–12}. But increasing confidence in the detection of a growing anthropogenic signal is inevitable and is not central to our confidence in climate predictions. This is founded on the ability of models to reproduce the present climate, including phenomena such as El Niño, together with our understanding of the model physics. Svante Arrhenius in the nineteenth century correctly estimated the global warming due to carbon dioxide to within a factor of two, and scientists have been refining this estimate ever since. Modern climate models contain many feedbacks that modify direct radiative forcing by greenhouse gases, but nobody has so far proposed a convincing mechanism large enough to balance this forcing effectively.

The bottleneck in the development of an effective climate policy is not so much the uncertainty of climate predictions as our lack of understanding of the interactions of climate change, the environment and the global socioeconomic system. Even so, the climate system has long response times, so we can afford to tune our control measures gradually (which does not imply that we can afford a period of inaction because the political process and economic restructuring also take a long time). By maintaining present progress in basic climate research, scientists can provide more reliable predictions on a time-scale compatible with the gradual implementation of policy.

Research and policy

From the simplified perspective of a climate scientist, climate policy reduces to a trade-off between two opposing human activities: climate change due to greenhouse-gas emissions, and the regulation of climate change through appropriate control measures. This poses three problems.

First, trade-off works only if the impacts of both activities are expressed in the same units, say dollars. But neither climate damages nor abatement costs are yet well

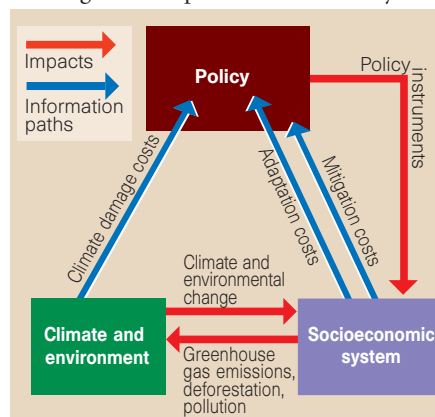


Figure 1 Schematic structure and interactions of integrated assessment models.

quantified — one cannot simply apply the laws of the marketplace. Factors such as species protection, the environment, public health and the social cost of economic dislocations must be compared with economic measures such as building higher dykes or relocating agricultural production.

Second, both climate damages and abatement costs vary geographically as well as with different sectors of human activity. Arriving at an international policy for climate protection is a complex process involving many actors with divergent interests, and the outcome depends on their game strategy — from collusion in lobbies and cartels to various levels of cooperation leading to global agreements.

Third, the game is played largely in the dark. The main costs of climate damage will accrue many decades and even centuries in the future. The uncertainties of climate prediction pale compared with the impossibility of predicting economic and technological developments over this time.

To analyse the problem of transferring climate research into climate policy, we need to develop models of the interactions between the climate, social and economic subsystems of the complete coupled system (see Fig. 1). Researchers can help to build such 'integrated assessment' models by simplifying their climate models for optimization studies, without sacrificing important information about regional climate, extreme events, the seasonal cycle, sea-level change and so on. This will not be easy, but various promising reduction techniques have been explored^{13–15}. Similarly, complex global economic models need to be reduced for incorporation into integrated assessment models.

Figure 2 shows atmospheric carbon dioxide concentrations and global mean temperature changes computed for different emission cases using a basic integrated assessment model¹⁴: *a*, an IPCC-type business-as-usual (BAU) scenario (extended beyond the normal IPCC range of 100 years); *b*, an emission path frozen at the 1990 level; and *c–e*, three optimized emission paths, in which the sum of the time-integrated climate damage and abatement costs is minimized. In *c*, climate damage and abatement costs are discounted at two per cent a year, in accordance with standard economic discounting practice, whereas in *d* and *e*, only abatement costs are discounted. (Economists use discount rates to account for, among other factors, the lower initial investment needed to generate a given amount of capital in the future because of capital growth through interest¹⁶.) Cases *d* and *e* differ by inclusion (*d*) or exclusion (*e*) of economic inertia.

Several conclusions relevant to policy can be drawn from this simple simulation. First, BAU gives large increases in global mean temperature of around 10°C after several hundred years (at these extreme values the

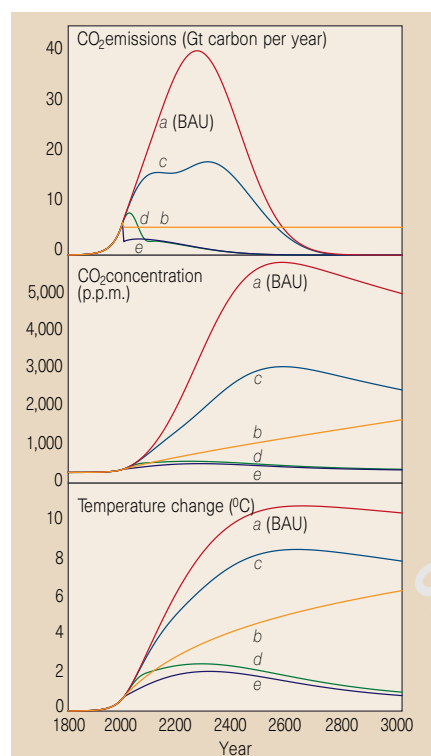


Figure 2 Carbon dioxide emission paths and concentrations and global mean temperatures for different emission scenarios (adapted from ref. 14, with a modified BAU scheme and an improved nonlinear climate response model).

climate model must be applied with caution, however, and indicates only the order of magnitude of climate change); the usual emphasis of climate policy on climate evolution over the next 50–100 years fails to address the far more severe long-term impacts of nonregulated emissions. Second, freezing emissions gains time for more restrictive regulatory measures later, but is insufficient in the long term. Third, the optimal solution for *c*, assuming the same economic discount rates for both cost terms, also leads to a large temperature increase, comparable with the BAU solution. Fourth, solutions consistent with sustainable development (*d* and *e*) are obtained only if climate damages are not greatly discounted. This is easily understood: if future climate damages are simply 'discounted', in the economic and colloquial sense, large climate damages in the distant future will be seen as acceptable in the optimized cost-benefit solution. The appropriate representation of the intertemporal cost relations for long-term climate change is central to the development of an effective climate protection strategy and the subject of considerable debate^{16–18}. Fifth, climate evolution in *d* and *e* is only weakly dependent in the long term on the regulatory measures implemented in the next two or three decades. In *d*, if we assume finite economic inertia, the emissions continue to follow the BAU path initially, whereas with no inertia they are adjusted downwards

immediately. But differences in long-term climate are small. Essential for limited greenhouse warming consistent with sustainable development is, rather, the transition to a fossil-free energy technology within one or two centuries.

Such simple models are intended not to provide quantitative data for specific policy decisions, but rather to identify general interrelationships for later quantitative analyses using more realistic models and indicate directions for model development.

These models fail to address, for example, the basic multi-actor nature of climate policy and the problems of decision-making under uncertainty. A realistic mitigation strategy must consider the different contributions to greenhouse warming, vulnerability to climate change and economic development of the different actors. It is also clearly unrealistic to define an optimal carbon dioxide emission path over many centuries, even though this time-scale is imposed by the long memory of the climate system. Climate policy requires a series of small steps conceived with a long-term perspective, constantly adjusted in the light of new technological and scientific developments.

Integrated assessment models, generalized for multi-actor interactions and uncertainty, should assist in this process. It is also worth testing whether game theory¹⁹ and the Bayesian approach to decision-making under uncertainty²⁰ can be applied to their development. Modelling will never replace the intricacies of international negotiations or modify the fundamental forces of divergent political interests, but it could guide negotiators towards an effective long-term strategy for climate mitigation. □

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The great debate on CO₂ emissions

Kilaparti Ramakrishna

There is a broad consensus that emissions of greenhouse gases, principally CO₂, must be reduced to minimize any adverse effects of climate change. But fierce argument attends the question 'How quickly?'.

Over the past few years, almost all of the world's leaders have acknowledged that the science telling us of long-term climate change, driven by anthropogenic emissions of greenhouse gases, is compelling, and that action is needed to reduce such emissions. In just over a week, on 1 December, parties to the United Nations Framework Convention on Climate Change of 1992 (FCCC) will convene in Kyoto* to agree upon and formally adopt the elements of just such an action. The intended outcome is legally binding commitments for industrialized (Annex I) countries and some provision to ensure that developing (non-Annex I) countries will engage in the process in the not-too-distant future.

There are, however, very different hopes and fears for what will come out of the Kyoto meeting. Leading observers from the environmental community and representatives of 'green' business groups who have followed the eight previous negotiating sessions, held over the two-and-a-half years preparatory to Kyoto, are not very optimistic that a thorough-going agreement to drive down emissions will emerge. If failure is inevitable, they hope that the conference will fail spectacularly. This is an all-or-nothing view, which holds that a new instrument that gives countries more time to comply with the existing commitments under the FCCC is as bad as the 'business as usual' scenario that has countries doing nothing to abate emissions. Spectacular failure, runs the argument, would result in full renegotiation.

In his paper on page 267 of this issue¹, Wigley gives a very different view, one which will encourage those who believe in conducting 'business as usual', at least for some years. This is a careful analysis, in which he outlines ways and timetables for developing countries to participate in emission reduction, set against different schemes of Annex I CO₂ abatement. But the paper has to be read with a crucial caveat in mind.

The analysis is based on a target of stabilizing atmospheric concentrations of CO₂ at roughly double the pre-industrial level — that is, at 550 parts per million by volume (p.p.m.v.). The justification advanced is that

this figure is in the centre of the 350–750 p.p.m.v. range that has been emphasized in scholarly studies, and that a firmer target has yet to be specified. However, it is far from clear what the target concentration should be. Azar and Rodhe² suggest, for example, that greenhouse-gas concentrations should be held to 450 p.p.m.v. CO₂ equivalent (CO₂, plus other greenhouse gases such as methane and nitrous oxide), or less, to have a reasonable probability of avoiding dangerous climate change. But even at 550 p.p.m.v., assuming that such a figure meets the objective of the FCCC, Wigley's view that the burden to be shared between Annex I and non-Annex I countries could require Annex I countries to do no more than continue with 'business as usual' until the year 2010, and non-Annex I countries to do the same until 2030, is highly contentious (and to my mind misguided).

Now, Wigley may not have wished to give the impression that delay in reducing emissions is a desirable course. But there are those engaged in the negotiations who would like to see nothing done at Kyoto (for instance, the business lobbies in the United States that have spent \$13 million in a mere two months to persuade people that abatement is both unnecessary and expensive). By overlooking the details, they will come away with just such a reading of Wigley's paper.

Leaving aside the immediate concerns dictated by Kyoto, such as adopting a legally binding agreement to strengthen the commitments of Annex I countries, the more serious question that this paper begs is not whether early action to reduce emissions will help (at two places in his paper, Wigley does state that it would), but whether delay in adopting implementable policies would be seriously damaging in economic terms. Again, the paper does not say that immediate practical action to reduce emission levels will be severely damaging economically; but it lends credence to all those who use such arguments.

This analysis should be read in conjunction with an earlier paper by Wigley, Richels and Edmonds³. There, the authors suggest that, issues of environmental damage aside, letting emissions rise in the short term before cutting them subsequently will be cheaper.

Taking the two papers together, an unmistakable message emerges — that there is no urgency about emission abatement. The argument here is this. Delaying measures until technology develops to a stage where energy efficiency improvements occur in great increments would obviate costly early action that will result in loss of jobs and debilitating economic effects. In other words, this viewpoint holds that delaying now will give those engaged in reducing greenhouse-gas emissions opportunities to reduce them swiftly later on.

Even if one ignores environmental issues, however, there is no consensus that delaying emission abatement is the cheaper route to stabilization of CO₂. For instance, a study⁴ sponsored by the US Department of Energy concluded that the United States "could reduce annual emissions of carbon dioxide and other greenhouse gases by as much as 20% from the 1990 level by 2010 at no net cost to the economy". Also, a group of distinguished US economists⁵ have pointed out that "there are many potential policies to reduce greenhouse gas emissions for which the total benefits outweigh the total costs" and that "there are policy options that would slow climate change without harming American living standards and these measures may in fact improve US productivity in the longer run".

Here, then, are powerful voices who say that early steps to reduce emissions in the United States are both warranted and need not be painful economically.

But even if one ignores these voices, and assumes that heavy costs are associated with immediately reducing greenhouse-gas emissions, there are still good reasons for early action. One is legal, as enshrined in a principle in the FCCC, which states that parties to the convention are to take "precautionary measures to anticipate, prevent or minimize the causes of climate change and mitigate its adverse effects". Another is scientific. Take the Dutch IMAGE 2 model⁶, which consists of three fully linked sub-systems (energy–industry, terrestrial environment, and atmosphere–ocean), unlike other models which concentrate on single aspects or spatial scales. The IMAGE 2 analysis showed that early action is critical when taking environmental and economic goals into consideration. As the authors pointed out, "if global emissions are too high in the year 2010, there is little chance afterwards to make a course correction which avoids climate-related impacts".

What, though, are the chances of our being able to make such a course correction? According to Ha-Duong, Grubb and Hourcade, the authors of another paper in this issue⁷ (page 270), they are low. Ha-Duong *et al.* observe that integrated assessment models under-represent socioeconomic inertia, the time lag between deciding to take

*See other contributions to this topic in Briefing on page 215 and Commentary on page 225.

action to reduce emissions and actually doing so; moreover, they consider that “higher adjustment costs” to correct the situation make it best to spread emission reduction over several generations. Their bottom line is that if we acknowledge that there is a significant risk that we need to stay below a particular level, then, given the socioeconomic inertia in energy systems, delay in abatement will prove costly. So Ha-Duong and colleagues’ conclusion is that there is a pressing case for limiting CO₂ emissions now.

In all, this is far more than a scholarly debate between proponents of ‘act now’ with practical action to abate emissions, or ‘delay until later’. What countries agree at Kyoto and then proceed to do will have a profound impact on the various possible future outcomes discussed by Wigley and Ha-Duong *et al.* If Annex I countries now take on legally binding commitments to abatement, it will send a strong signal to the private sector of the necessity of investing in cleaner energy sources. Moreover, the non-

Annex I countries that are beginning to question the commitment of industrialized nations to greenhouse-gas reductions will be reassured that it is not a problem that only they need to cope with. In the end, the combined action of government regulation and private-sector involvement in Annex I countries will give a powerful impetus to non-Annex I countries to adopt similar emission-reduction measures. □

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Developmental biology

The worm keeps turning

Bruce Bowerman

Who escapes the lurking serpent’s mortal sting? Not he that sets his foot upon her back. Even the smallest of worms will turn, when trodden on¹.

The roundworm *Caenorhabditis elegans* is one of the most important models for the study of animal development. It is unique in that the generation of all 558 cells of a newly hatched roundworm larva from a few embryonic founder cells is invariant. The description of this process, a monumental effort undertaken by Sulston and colleagues², required seven years of patient peering through a microscope, repeatedly observing embryonic cell divisions and carefully recording the positions of nuclei — as small as 1 µm in diameter — in hundreds of embryos. The way that the embryonic founder cells generate all the different cell lineages of an entire roundworm is truly invariant: every division axis, every slight

asymmetry in cell size and every contact made with another cell is indistinguishable from one embryo to the next, with very few exceptions. On page 294 of this issue Kaletta *et al.*³ provide the first genetic evidence that a single mechanism may operate throughout the *C. elegans* embryo to maintain the invariance of all cell lineages.

The embryonic cells of *C. elegans* not only make studies of development at a single-cell resolution possible but also pose an intellectual challenge. The one-cell-stage embryo undergoes a series of asymmetric and asynchronous divisions to generate five somatic founder cells, each of which subsequently divides to produce descendants that together form all the somatic cells of the roundworm larva. Two of these founder cells, D and E, each give rise to either 20 muscle or 20 gut cells, respectively, whereas another three — AB, C and MS — produce the remaining 518 somatic cells that have many different fates,

constituting all tissues apart from the germ line. It is difficult to imagine how the lineages derived from these three founder cells can be so invariant.

Ironically, Sulston’s conclusion that the embryonic lineage in *C. elegans* is invariant led even most ‘worm people’ to dismiss the roundworm embryo as an extreme case of lineage-dependent development, seemingly irrelevant to more complex animals, where the generation of a large number of cell lineages that vary from embryo to embryo depends on intricate interactions between cells, and complex signalling pathways, rather than a predetermined destiny.

In 1987, however, this smallest of worms first turned for Jim Priess, an early disciple who found the embryo appealing in its elegant simplicity. Not impressed with the logic that invariance implies irrelevance, he interchanged the positions of the two daughters of the AB founder cell (AB for anterior blastomere), which produces 389 of the 558 cells in a hatched larva⁴. To everyone’s surprise the rearranged embryo developed normally in spite of the ultimately very different fates of the two AB daughters. This one simple experiment conclusively demonstrated that more than half the embryo depends extensively on cell interactions for the development of different lineages. Subsequent genetic and molecular studies showed that widely conserved cell-signalling pathways are required to properly specify some of the most complex lineages in the *C. elegans* embryo⁵, deepening even further the mystery of invariance.

Now the worm has turned yet again, this time in the laboratory of Ralf and Heinke Schnabel, who, with Titus Kaletta, show that eliminating a gene called *lit-1* results in mutant roundworm embryos with massive lineage defects³. The first defect becomes apparent at the 4-cell stage, where the parent of two somatic founder cells divides to produce a posterior daughter called E that makes gut (endoderm) and an anterior daughter called MS that makes body-wall muscle and pharyngeal cells (mesoderm). In *lit-1* mutant embryos, both daughter cells adopt an anterior fate (see Fig. 1). This results in an excess of mesoderm and a complete loss of intestine (hence the gene name *lit-1*).

The investigators go on to show that in these mutant worms many embryonic cells that divide along the anterior–posterior axis similarly produce daughters that adopt anterior fates at the expense of posterior fates. These posterior-to-anterior fate transformations are observed in lineages that develop without the help of other cells (autonomously), and in lineages that require signals from other cells. For example, during normal development the MS founder

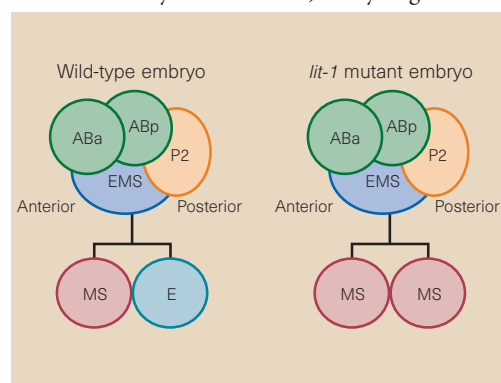


Figure 1 Transformation of embryonic cells from a posterior to an anterior fate in *lit-1* mutant embryos. The first lineage defect in *lit-1* mutant embryos occurs at the 4-cell stage. In wild-type embryos the most ventral blastomere EMS becomes polarized to produce a posterior daughter called E and an anterior daughter called MS. E makes the intestine (endoderm) whereas MS makes body-wall muscle and pharynx (mesoderm). In *lit-1* mutant embryos, the polarization of EMS is lost and both daughters adopt the anterior MS fate.

autonomously generates the posterior part of the pharynx (the neuromuscular mouth and throat of the worm), and AB descendants require a signal from MS to produce the anterior part of the pharynx⁴. In *lit-1* mutants, both the MS and AB lineages that contribute to the pharynx exhibit transformations in their ultimate fate.

Posterior–anterior transformations in cell fate are seen as early as the third round of embryonic cell division in *lit-1* mutants, when E and MS first appear, and continue with each round of division until as late as the eighth round. Indeed, the transformation of a cell with a posterior cell fate to one with an anterior cell fate occurs repeatedly with each division in the lineages generated by all founder cells except D, which only produces muscle.

The *lit-1* gene has yet to be identified, but the mutant phenotype in *C. elegans* suggests that it plays a fundamental role in specifying whether a cell will have an anterior or posterior fate. In the absence of the *lit-1* gene, both daughters of an anterior–posterior cell division adopt an anterior fate, whereas when this gene is expressed cell division yields one daughter with an anterior fate and one daughter with a posterior fate (a process called binary switching).

Previous studies have defined two genetic networks in *C. elegans* that generate anterior–posterior polarity in embryonic cells. Six *par* genes (for partitioning-defective) are required to polarize the one-cell-stage zygote along its anterior–posterior axis⁶, apparently in response to the entry of sperm into the egg⁷. In addition, the widely conserved *Wnt* (wingless) signal-transduction pathway (which is vital for normal development in *Drosophila* and in mice) is required to polarize the 4-cell-stage parent of E and MS along its anterior–posterior axis^{8–10}. Some of the fate transformations seen in *lit-1* mutant embryos resemble those in worms that have mutations in *Wnt* pathway genes, whereas other *lit-1* defects appear absent in *Wnt* pathway mutant embryos. Thus, *lit-1* may join *par* and *Wnt* as another system for specifying anterior–posterior polarity. The close similarity between some *par* genes^{11,12} and all *Wnt* genes¹³ among most organisms suggests that *lit-1* may also be highly conserved. Alternatively, *lit-1* could represent a relatively phylum-specific mode of developmental regulation. A fundamental issue for future studies of embryogenesis in *C. elegans* will be to determine if and how these three different anterior–posterior polarity systems interact. Pretty complicated for a little worm — stayed tuned for further squirmings. □

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Colossal magnetoresistance

Not just a load of bolometers

Neil Mathur

Magnetoresistive materials have properties that can change radically under magnetic fields; and they promise much to science and technology. The cubic manganese perovskites and other such exotic materials were discussed at meetings last month in London* and Sheffield†. A deeper understanding emerged of the complex and subtle science behind their properties, as did a cheap and practical means of sensing small magnetic fields, and a new material for infrared detectors.

Large changes in the electrical resistivity of a material may be induced by a magnetic field, especially if the material has a magnetic structure that interacts strongly with its electronic structure. ‘Magnetoresistance’ (MR) effects of this type can be as large as a million per cent¹ in ‘colossal magnetoresistance’ (CMR) perovskites such as $\text{La}_{0.7}\text{Ca}_{0.3}\text{MnO}_3$. A decrease in resistance correlates with the alignment of the magnetic moments of the manganese ions², and the effect is largest when the moments can easily be aligned by a field. This is the case near the spontaneous alignment temperature T_C .

*Understanding and Utilising Colossal Magnetoresistive Materials, Royal Society, London, UK, 1–2 October 1997.

†3rd OXSEN Meeting, Sheffield University, UK, 3–4 October 1997.

Actually, a great deal of extra physics is required to explain the magnitude of the CMR effect. Here I concentrate on the perovskites. In the low-resistance state there are delocalized electrons, which move relatively freely under the laws of quantum mechanics, as they do in a metal. In the high-resistance state, current-carrying electrons instead get ‘bogged down’ on individual ions, and behave more like classical particles. When electrons move between manganese sites, they interconvert Mn^{3+} and Mn^{4+} . The Mn^{3+} ion is much larger than the Mn^{4+} ion, and therefore the transfer of an electron must be accompanied by a structural distortion of the surrounding lattice. That increases the resistance of the high-resistance state, making true CMR possible.

It appears that this structural distortion manifests itself as the displacement of the six oxygen atoms surrounding each manganese ion (A. J. Millis, Johns Hopkins Univ.). Nevertheless, a full match with the experimental data remains elusive. Nuclear-magnetic-resonance experiments remind us that nearby manganese moments remain aligned³ even well above T_C (C. Z. Kapusta, Krakow Univ.). Because each current-carrying electron interacts with this complex magnetic environment, a complete picture of the high-resistance state must take this

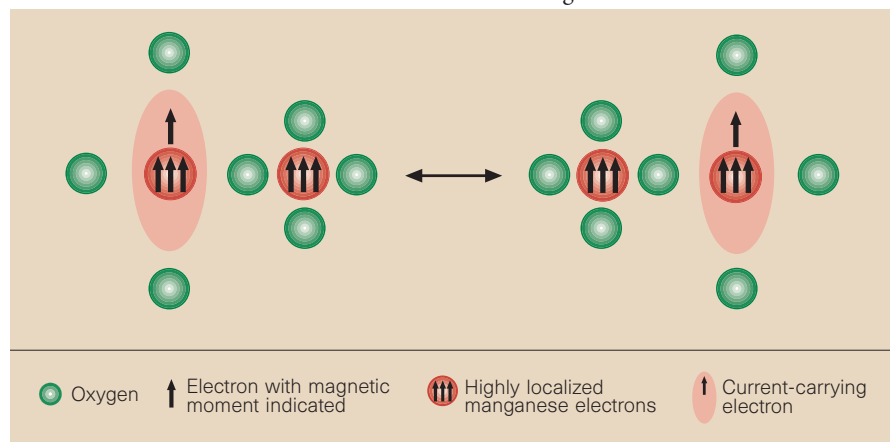


Figure 1 The high-resistance state in a colossal-magnetoresistance perovskite. There are three highly localized 3d electrons on each manganese site, which have parallel spins. Near and above the spontaneous aligning temperature, the movement of current-carrying electrons is liable to be impeded by structural and magnetic distortions. A magnetic field can align the magnetic moments on all sites, and so allow the current-carrying electrons to move freely.

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into account or the case is far from solved.

Clearly, there is an intriguing interdependence between the magnetic, electronic and physical structure of a typical perovskite CMR material. Macroscopic strain can therefore radically alter magnetic and electronic properties. Spectacular changes can be produced using hydrostatic pressure or partial chemical substitutions (J. Fontcuberta, ICMB, Barcelona), or thermodynamically induced structural phase transitions (D. McK. Paul, Warwick Univ.).

To produce the bulk behaviour described above, huge magnetic fields of several tesla are needed — too large for most commercial applications. But a defect structure, or heterostructure, can respond to changes in magnetic field as small as those sampled by the read head in a disk drive. There are some promising examples of multilayer thin-film heterostructures (J. Sun, IBM; M. R. J. Gibbs, Sheffield Univ.), as well as defect structures such as grain boundaries in polycrystalline samples (S.-W. Cheong, Bell Labs) and, in an area of my own work, thin-film artificial-grain-boundary devices (J. E. Evetts, Univ. Cambridge).

A new, cheap and practical way of fabricating sensors of small magnetic fields is to employ a powder, as a large surface area constitutes a large quantity of defect material (J. M. D. Coey, Trinity Coll., Dublin; S.-W. Cheong). 'Powder magnetoresistance' was demonstrated in compacted powders containing the metallic CrO_2 particles that are found in magnetic recording media. These have a low-field MR behaviour similar to typical CMR materials containing defects. Tuning the relative amounts of CrO_2 and some inert material gives plenty of scope for engineering a range of desired responses.

To stimulate an understanding of the effect of grain boundaries on the CMR response, the phenomenological 'mesoscale magnetoresistance' model (MMR) was presented (J. E. Evetts; N. D. Mathur), alongside a microscopic theoretical model (G. A. Gehring, Sheffield Univ.). Both models conclude that antiferromagnetic interactions depress magnetic order near a grain boundary, and so leave it susceptible to small changes in magnetic field. However, neither model takes into account the 'width' of this interfacial region. The next step may involve treating it as an inhomogeneous entity.

Pyrochlores also have CMR, but the underlying physics is different in these low-carrier-density materials, and they may be simpler to understand than the perovskites. In particular, the magnetic structure of, say, $\text{Ti}_2\text{Mn}_2\text{O}_7$ originates in the Mn–O sublattice and is therefore physically distinct from the electronic structure, which originates in the Ti–O sublattice⁴. Furthermore, there is only one 'size' of manganese ion present, so the influence of the physical structure is reduced. The magnitude of the pyrochlore

CMR response may be understood by taking into account the alignment of nearby manganese moments above T_C (P. B. Littlewood, Univ. Cambridge) — a matter relevant to the perovskites. Pyrochlores may also be more suitable⁵ than perovskites for low-field sensor applications: grain boundaries in $\text{Ti}_2\text{Mn}_2\text{O}_7$ display a fairly temperature-independent MR response below T_C .

What immediate uses are there for the CMR materials? Although no suitable niche for CMR sensors of magnetic fields has yet been established, there is a use for the perovskites that exploits the often steep resistance–temperature curve near T_C . Arrays of elements held at this temperature would be very sensitive to the arrival of infrared photons, and are set to become the active elements in infrared cameras, or bolometers (T. Venkatesan, Neocera Inc. & Univ. Maryland).

Ecology

Feeding patterns on forest floors

Peter D. Moore

There are many reasons why patterns emerge in the arrangement of individuals in a population. The environment may be patchy, providing a mosaic template for the patterns of populations; individuals may interact with their neighbours — both positively and negatively — to create non-random arrangements; or dispersion may reflect historical processes, dispersal capacity or the presence of barriers. In the case of plants, the static nature of which lends itself to the study of patterns, arrangements can also arise from the activities of more mobile organisms that consume fruits and, in so doing, disperse seeds.

According to two reports in the *Journal of Ecology*, the complexity of patterns among some plant populations in tropical rainforests may result from the behaviour and habits of their mammalian predators. The howler monkeys of French Guiana (studied by C. Julliot)¹ and the tapirs of Brazil (J. M. Fragaso)² are both currently under scrutiny as possible causative agents in the generation of plant patterns in South American rainforests.

Pattern in a plant population is essentially a departure from randomness in the dispersion of the individuals. The involvement of animals in the creation of these patterns is certainly not a new idea — dietary preferences in grazing animals, the engineering activities of beavers, the wallowing of peccaries, and the catastrophic disturbance by foraging of wild pigs can all create patches in the environment which support distinctive vegetation. But the direct effect of vertebrate herbivores on the dispersal and survival of seeds influences the final spatial arrangement

So, even though it is the perovskites that are currently arousing technological interest, a full understanding of the CMR phenomenon may come first in the pyrochlores, which appear simpler. CMR is also seen in layered perovskites⁶, chalcogenide spinels⁷ (such as CeFe_2S_4) and silver chalcogenides⁸ (such as Ag_{2+x}Se) — so it would be false to say that there's only one CMR. □

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Figure 1 By ingesting seeds and excreting them some distance from the 'parent' plant, animals can influence the pattern of plants on the forest floor. Julliot¹ and Fragaso² have found that red howler monkeys (*Alouatta seniculus*; top) and tapirs (*Tapirus terrestris*) increase the chances of survival of the ingested seeds because they remove them from the high concentrations of predators (such as beetles) around the parent plants.

of mature plants in a much more subtle way.

In 1970, Janzen³ put forward a basic model to describe the impact of beetle predators upon seeds in a forest: if the initial seed fallout is assumed to be densest in the immediate vicinity of a tree and to decline as

one moves away from the source, then a seed predator would most easily make a living — and, therefore, reproduce fastest and consume seed most efficiently — close to the tree. Finding seeds would be more difficult with increasing distance from the tree, so the population of foraging predators would be lower and the chances of some seeds escaping detection and surviving would rise. According to this model, trees subject to beetle seed-predation should be immediately surrounded by an umbra in which the probability of survival is low. Beyond this will be a penumbra, determined by the dispersal capacity of the seeds, where seed density may be lower but the survival probability of any seed arriving there is increased.

If this model is valid, it should result in wide spacing between individuals of a given species, and it should operate against any tendency to form clumps. In practice, the Janzen model has often been shown to apply convincingly to many rainforest tree species. But some forest plants occur in clumps, and a selection of these has been described and studied by Julliot in French Guiana¹. In an area of primary, evergreen rainforest, with a continuous canopy at 30–40 m and emergent trees to 65 m, Julliot studied a troop of six red howler monkeys (*Alouatta seniculus*). These monkeys are strictly vegetarian in diet, and they consume leaves and fruits from a range of species. Concentrating on six of the more important plant species that contribute to this diet, Julliot found that the forest floor beneath the regular dormitory sites of the howler troop supported four times the density of seedlings found elsewhere. The monkeys were responsible for dispersal of the seeds through their guts, depositing some 40 per cent of their faecal material while they were in their sleeping quarters. So, in this case, a mobile vector in which seeds are gut-transported can result in clumped dispersion (which is apparent when sampled using plots 32 m × 40 m). One might anticipate that such local seed density could result in the same concentrations of invertebrate predators as found beneath a parent tree, but the fact that seedlings (rather than seeds) were counted in this study shows that many of the seeds survived beyond germination.

A similar story emerged from Fragaso's study² of the population dynamics and pattern in the palm *Maximiliana miripa*, which grows in the northern part of the Amazonian rainforest in Brazil. This time, the dispersal agent is the tapir (*Tapirus terrestris*), which readily consumes the large fleshy fruits of *Maximiliana*. The seeds pass through the gut undamaged and are deposited in specific latrine sites, almost always at the base of tauri trees (*Couratari multiflora*). These sites are used regularly over long periods and, on average, almost 1,000 *Maximiliana* seeds were found per site.

Bruchid beetles attack the seeds on the

ground, and Fragaso found that 77 per cent of the seeds beneath the parent trees were rendered non-viable by these invertebrates, whereas fewer than one per cent were killed by beetles in the latrine sites. Rodents acted as secondary dispersers of seeds from the latrines, usually carrying them a maximum of 5 m before burying them in hoards. So, tapirs seem to remove many palm-tree seeds from the centres of beetle predation, providing the seeds with the opportunity to form new clusters of *Maximiliana* in the vicinity of the tapir latrines.

Gorillas, lemurs, baboons and rhinoceroses have all been implicated in the dispersal of seeds in tropical forests. Howler monkeys and tapirs can now be added to this list. Quite

apart from the interest of these studies in terms of creating habitat diversity and plant spatial-patterns within tropical rainforest, the complexity of relationships among dispersers and seed predators could be vital to the recovery of a forest after disturbance. Such trees as *Maximiliana*, for example, can act as secondary colonists of cleared areas. So, by maintaining viable populations of its seed vector, a forest could re-establish itself much more quickly. □

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Archaeology

Magnetic trace of a giant henge

Elizabeth Aveling

A new survey at Stanton Drew in Somerset, UK, has revealed a previously unknown 5,000-year-old henge ditch surrounding the Great Circle (the second largest stone circle in Britain, after Avebury), and inside that a series of concentric rings, probably the remains of the largest wooden henge yet discovered. This has been done without any excavation — instead, the archaeologists measured the magnetic susceptibility of the soil.

Magnetic susceptibility is the ability of a material to become magnetized by a magnetic field (such as the Earth's). Archaeological features may have a higher or lower magnetic susceptibility than the surrounding soil, and such variations can be detected using a magnetometer. Many archaeological sites have been surveyed in this way.

Anticipating that Stanton Drew would show only weak magnetic anomalies, the team from the Ancient Monuments Laboratory of English Heritage adapted their survey to make it as sensitive as possible. A narrow sampling interval was used, and their magnetometer was modified so that the lower sensor was as close to the ground as possible.

Their most remarkable discovery was the presence of nine concentric rings of weak magnetic anomalies within the Great Circle. These are not additional ditches, but a series of closely spaced yet isolated anomalies, probably the remnants of rings of post holes. Although wood only possesses a very small magnetic susceptibility (and the wooden posts have long since disappeared), there are reasons why post holes might give positive magnetic anomalies. Magnetotactic bacteria that thrive on rotting wood could lead to concentrations of biogenic magnetite in the soil, which remain long after the wood has decayed¹. Experiments have shown that the remains of wooden posts can indeed be detected by magnetic surveying².

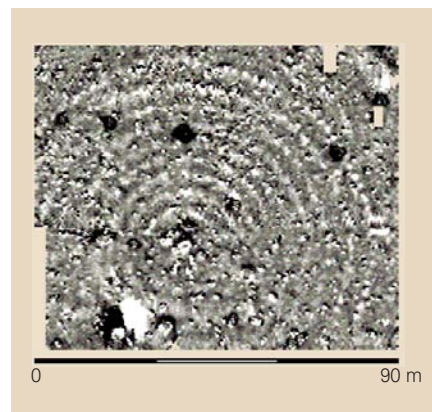


Figure 1 The newly discovered wooden henge of Stanton Drew. This magnetic map shows nine huge rings, tracing part of an elaborate structure that is about 5,000 years old.

Alternatively, burnt deposits (timber or otherwise) in the post holes would also produce a variation in magnetic susceptibility, as would a back-fill of topsoil (which tends to have a greater magnetic susceptibility than the underlying soil layers).

There have been no excavations at Stanton Drew, and so there is no secure evidence that the magnetic anomalies are post holes — but based on evidence from analogous sites such as Durrington Walls³ and Woodhenge⁴, it seems a safe assumption. Stanton Drew bears some resemblance to these sites, but it is twice as large as any of the seven other known timber complexes (which are peculiar to Britain), and so it is an important find. Its use might have been ceremonial, perhaps as the focal point for a certain tribe; there is no evidence yet of any astronomical purpose.

English Heritage is currently experimenting with a new form of magnetometer. Their caesium alkali vapour magnetometer has a sensitivity of 0.01 nanotesla (less than a

millionth of the strength of the Earth's field) and it has been used to repeat parts of the Stanton Drew survey. Preliminary results show that this instrument has potential for 'seeing' archaeological features in even more detail.

The Stanton Drew survey is a fine example of the use of magnetic surveying in archaeology, and the power of the technique to detect features that would otherwise go unnoticed. □

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Fluid dynamics

Diffusion in a different direction

David A. Weitz

Diffusion of particles or molecules in a fluid is an essential manifestation of thermal energy. It is seen in the familiar brownian motion of dust particles in a fluid or a gas, and it ensures the mixing of different molecules in a fluid. So mixing, at the shortest length scales, results from diffusion rather than convection. This is behind a standard method for measuring molecular diffusion coefficients: a sharp concentration gradient is established between two fluids, and the decay of this gradient as the two fluids mix determines the diffusion coefficient of one fluid in the second. Observers look in a direction perpendicular to the gradient (that is, with the interface edge-on), and the results are interpreted assuming a smooth relaxation of the concentration gradient. But that may not be valid: on page 262 of this issue¹, a team from Milan report unexpected spatial fluctuations in the concentration of two fluids mixing by diffusion.

The authors, Vailati and Giglio, take the unorthodox approach of looking along the direction of the gradient, so that no large-scale variation from the main concentration profile is seen. Rather than the expected uniform variation in concentration, they observe huge spatial fluctuations of the concentration perpendicular to the gradient. These fluctuations develop rapidly, and persist until the gradient has been completely relaxed. It is only by looking from this new perspective that they can observe these surprising fluctuations.

The authors use two fluids that can be placed, by a suitable choice of temperature, near a 'consolution critical point', at which they go from miscible to immiscible. The temperature is first lowered, making the fluids separate, with the heavier fluid on the bottom. It is then quickly raised above the critical temperature, making the fluids miscible. Diffusion relaxes the concentration

gradient — but it also creates enormous fluctuations, in a direction perpendicular to the gradient, which are large enough to be seen from the variations they cause in optical phase change and in the pattern of scattered light.

Where do these fluctuations come from? The authors propose that naturally occurring velocity fluctuations transport small volumes of fluid along the gradient, which then relax most quickly by diffusion, leading to large-scale spatial fluctuations in the concentration perpendicular to the gradient (Fig. 1a).

In retrospect, it is perhaps not so surprising that a diffusive process can be so dominated by fluctuations. Diffusion is simply the continuum limit of a statistically random process, and if the large statistical fluctuations grow faster than any relaxation process, then highly disordered patterns can result. An example is the diffusion-limited accretion of particles onto a growing seed². If the growth kinetics are controlled by diffusion, and if the particles are not allowed to relax once they strike the surface but instead are pinned onto the point of first contact, then they form highly disordered structures.

The structures produced by diffusion-limited aggregation are fractals, that is, they are scale invariant. They produce a light-scattering intensity that is similar in form to that from the diffusive fluctuations reported by Vailati and Giglio. In both cases, the scattering intensity is a power-law function of the scattering wave vector, reflecting the lack of any characteristic length scale. In both cases, a simple solution of the diffusion equation would lead to a very uniform structure. In both cases, the fluctuations dominate because they cannot relax quickly enough.

The fluctuations observed by Vailati and Giglio are limited in size only by gravity: if the volume transported by the velocity fluctuation is too large, then it is relaxed by convection due to buoyancy, rather than by diffusion — the displaced volume returns to its original location without contributing to the spatial fluctuations (Fig. 1b).

Traditional measurements do not have this unusual perspective, explaining why these fluctuations are typically not observed in diffusion coefficient measurements. More generally, the reason they have escaped our observation until now is probably that mixing is controlled by diffusion only on short length scales. On larger scales, either convection or stirring usually dominates.

By using buoyancy-matched fluids, the scale of the fluctuations could be greatly increased. Even more pronounced effects would occur if the experiment were carried out in space, in microgravity. Then, the length scale of the fluctuations could become truly macroscopic, easily approaching a millimetre. This would have profound consequences. It would radically alter any

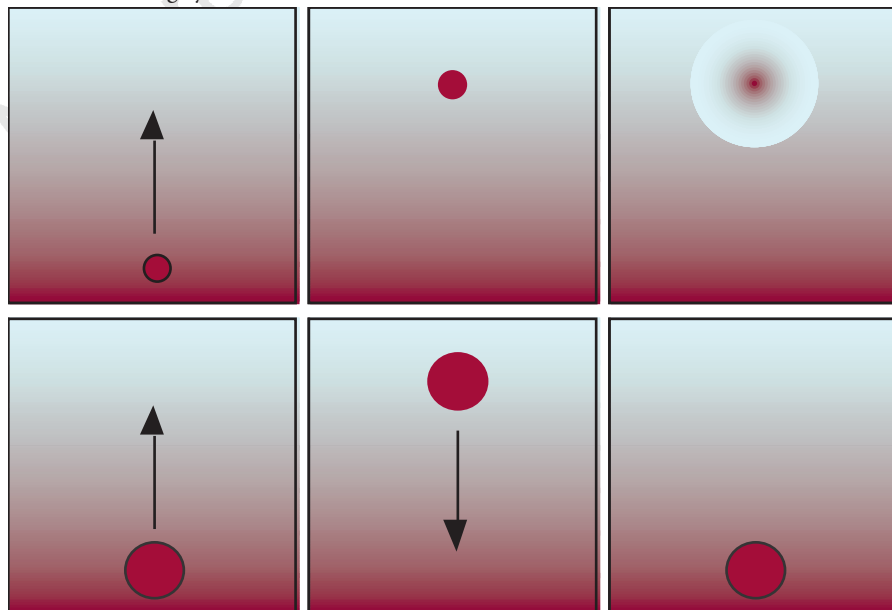


Figure 1 How large fluctuations can occur in diffusion. a, A velocity fluctuation carries a small volume of one fluid into the other, along the direction of the concentration gradient. It relaxes by diffusion, leading to a large spatial fluctuation perpendicular to the gradient. b, Gravity limits the size of the fluctuations, because larger volumes do not have time to diffuse before buoyancy brings them back to their original position.

measurements that depend on diffusive motion — measurements of particle sizes and diffusion coefficients, for example, and of the behaviour of critical fluids. Indeed, any fluid measurements performed in space must be interpreted very carefully, to fully take into account the effects of the fluctuations.

Moreover, some manifestations of diffusive motion that are well established on Earth may be profoundly changed in microgravity.

Virology

Illicit viral DNA

Robin A. Weiss and Paul Kellam

More than 20 years ago, the late Victor Zhdanov at the Ivanosky Institute of Virology in Moscow published a remarkable paper¹ claiming that complementary DNA copies of RNA viruses such as measles and polio occurred in retrovirus-infected cells. The observations raised eyebrows at the time, because promiscuous cDNA synthesis seemed to run counter to everything known about viral replication. But the data were neither confirmed nor refuted, and were soon forgotten.

On page 298 of this issue², Rolf Zinkernagel's group resurrects the issue posed by Zhdanov's results. Klenerman *et al.*² show that cDNA fragments of the RNA-replicating lymphocytic choriomeningitis virus (LCMV) form in mice and in murine and hamster cells in culture which express retroviral reverse transcriptase, the enzyme which uses an RNA template to synthesize DNA. The formation of LCMV cDNA is inhibited by azidothymidine (AZT), confirming that reverse transcriptase activity is involved. There is, of course, ample evidence for reverse transcription having occurred in evolution, with the formation of processed DNA or pseudogenes from RNA³. But the LCMV cDNA is a case of a non-retroviral RNA being caught red-handed in the act of seemingly illicit DNA synthesis.

Many viruses of animals, plants and bacteria carry their genetic information in the form of RNA. With the exception of retroviruses, they replicate through RNA intermediates, so that no viral DNA sequences are synthesized at any stage of the viral life cycle. LCMV is such a virus⁴ (and belongs to the arenavirus family, which also includes the dreaded Lassa fever virus). The RNA in LCMV virus particles is composed of two 'ambisense' single-stranded molecules, large and small. Part of each molecule has the same coding sense as messenger RNA from which the viral proteins are translated, and part is complementary to mRNA. LCMV encodes and packages an RNA polymerase which makes complementary RNA from the genomic RNA template. The viral polymerase is not known to have reverse tran-

Even the mixing of fluids may be appreciably affected, by a change in the timescale of homogeneous mixing. Further surprises are probably to be found in diffusion-driven phenomena without gravity. □

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scriptase activity. That is why it is so surprising that Klenerman *et al.* detect DNA sequences homologous to viral RNA.

When outlandish claims are based on detection through the polymerase chain reaction (PCR), the sceptic's initial reaction is to suspect false-positive data. After all, a laboratory in which cloned viral sequences are handled, or where reverse-transcriptase PCR is routinely used to detect viral RNA, is just the right environment for error, as we all know to our cost. But Klenerman *et al.* appear to have performed all the appropriate controls to guard against contamination.

One reason why they investigated the presence of cDNA was to try to solve an

immunological puzzle — mice which have cleared all evidence of previous LCMV infection continue to show strong immune responses as if some viral antigen persisted in the animals. The authors therefore speculate that the cDNA might act as a naturally produced form of DNA vaccine that produces antigen. This is difficult to conceive for cDNA lacking promoter sequences for expression. However, if any of the cDNA sequences were to integrate downstream from cellular gene promoters or within an open reading frame, a low level of specific peptides might be expressed that would be sufficient to load major histocompatibility antigens in antigen-presenting cells, and thereby elicit an immune response.

Klenerman *et al.*² detected LCMV cDNA in mouse and hamster cells expressing reverse transcriptase activity. But it was not found in a variety of other cells without such activity, or in reverse-transcriptase-positive guinea-pig cells. Further work is required to find out whether retroviral reverse transcriptase is responsible for the LCMV cDNA synthesis, or whether some other cellular component in mouse and hamster cells confers a reverse transcriptase activity upon the LCMV RNA polymerase. One of the reverse-transcriptase-negative cell lines should be infected with amphotropic murine leukaemia virus and then super-infected with LCMV to see whether cDNA

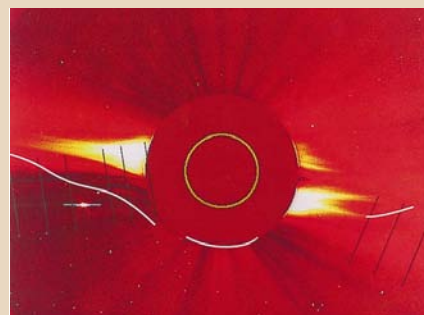
Solar physics

Galileo through the Sun's streamers

The Galileo satellite, on its tour of Jupiter's moons, has provided a remarkable bonus for solar physicists. In January, the Sun obstructed our line of sight to the distant probe. By monitoring radio signals from Galileo as they passed through the Sun's corona, astronomers have solved an old problem about the origin of the solar wind. It has long been known that the wind has two distinct components, slow and fast. But where on the surface of the Sun do they originate?

This image (Habbal, S. R. *et al.* *Astrophys. J.* **489**, L103–L106; 1997) is a white-light view obtained using the Solar and Heliospheric Observatory (SOHO). The solar disk is blanked out, revealing the hot, inner coronal regions and filamentary structures known as streamers. Jupiter is the bright point to the left of the solar disk.

Galileo's apparent passage behind the Sun is marked by the straight black lines, which show where the slit of a spectrometer on SOHO was positioned to make ultraviolet measurements of the corona, simultaneous with the radio transmissions. The UV spectra give a rough indication of the Solar wind speed,



and the 94 km s⁻¹ contour is shown in white. Scintillation of Galileo's radio signal also indicates wind speed, importantly with very high spatial resolution: on one occasion, the scintillation increased markedly (a sign of the slow wind), and, by no coincidence, a streamer stalk intercepted the line of sight to the probe at the same time. It seems that the slow wind comes from streamers, and the fast wind comes from the whole surface — not, as had been thought, only from the polar regions.

Eventually, these results may help solve the biggest mystery of the solar corona — the nature of its heating mechanism.

Karen Southwell

only appears in the presence of the enzyme.

If retroviral reverse transcriptase really is the culprit, how does the enzyme gain access to its illicit RNA template, and what primer is used to initiate reverse transcription? In retroviruses, various transfer RNA molecules act as primers by specifically annealing to primer binding sites in the viral genome. LCMV sequences could be analysed for potential primer binding sites or for sequences that may allow LCMV RNA to be packaged in retrovirus particles. This might explain why LCMV cDNA synthesis occurs only in some cells which are positive for reverse transcriptase, as the enzyme is not present in the cytosol in active form, and cross-packaging may be specific to certain types of retrovirus. Alternatively, reverse transcriptase might be incorporated into LCMV particles, which contain ribosomal 28S and 18S RNA, as well as low-molecular-weight 4–7S cellular components⁴ which may include tRNA.

A host of questions remains. How general might the phenomenon described by Klenerman *et al.*² be? Is it solely limited to retroviral reverse transcriptases, or does it encompass related polymerases of, say, the hepatitis B virus family? Is LCMV a special case because of cross-packaging of RNA or of reverse transcriptase, or is it a convenient marker RNA

sequence as no homologous DNA exists in uninfected cells? Was Zhdanov right after all? Will humans persistently infected with the retrovirus, human immunodeficiency virus, make cDNA forms of other, non-retroviral RNA viruses? Could cDNA synthesis help to explain why it is so technically difficult to detect negative-strand genomes of hepatitis C virus⁵ (as evidence for *de novo* replication) — if the 'false' positive reverse transcriptase PCR results that are so common was natural cDNA? How long are the cDNA transcripts of LCMV, and do they integrate into the host genome?

Clearly, there is much to keep molecular virologists occupied arising from these new observations. They present a phenomenon that might have consequences in understanding the reshuffling of genetic material, and the pathogenesis of double virus infections. □

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sequence as no homologous DNA exists in uninfected cells? Was Zhdanov right after all? Will humans persistently infected with the retrovirus, human immunodeficiency virus, make cDNA forms of other, non-retroviral RNA viruses? Could cDNA synthesis help to explain why it is so technically difficult to detect negative-strand genomes of hepatitis C virus⁵ (as evidence for *de novo* replication) — if the 'false' positive reverse transcriptase PCR results that are so common was natural cDNA? How long are the cDNA transcripts of LCMV, and do they integrate into the host genome?

This is not the first study to suggest that mutation rates are higher in males. A similar logic has been applied to the sequences of genes on the X and Y chromosomes of primates^{8,9} and rodents⁹. But the higher rate of change in Y-linked compared with X-linked genes does not unequivocally demonstrate a higher rate of mutation in males because there is an alternative explanation¹⁰: mutation rates reflect in part a trade-off between the benefits of a low mutation rate and the costs of the molecular machinery needed to ensure accurate replication. Mutations in genes on the paired sex chromosomes (X in mammals, Z in birds) are more disadvantageous than mutations in autosomal genes because deleterious recessive mutations will be exposed when hemizygous (that is, occurring on the X or Z chromosome opposite a non-coding Y or W chromosome) but not when heterozygous. So selection should favour more accurate replication, and hence a lower mutation rate, of the X or Z chromosome. In mammals, the two explanations — more mutations in the sex with more divisions in gametogenesis (the male), and fewer mutations on the paired sex chromosome — cannot be distinguished because they both predict higher rates of change in Y-linked than in X-linked genes. In birds, males are homogametic, so the higher mutation rate of *CHD-Z* compared with *CHD-W* can only be explained by a greater number of cell divisions in males.

If mutations do occur primarily during cell division, the relative mutation rates in males and in females should equal the relative numbers of cell divisions during spermatogenesis and oogenesis. This is not known in birds, but has been estimated in humans: there are about 33 divisions from zygote formation to oogenesis, and 205 from zygote formation to spermatogenesis (in a 20-year-old man)⁹. The ratio of these two numbers (6.2) is close to the ratio of the mutation rates in male and in female higher primates (6; ref. 9).

A higher mutation rate in males than in females implies that point mutations are largely associated with cell division and are not caused by other factors such as methylation or oxygen radicals that are unrelated to replication. This implication touches on diverse areas of evolutionary biology. First, it provides an explanation for the effect of generation time on the rate of molecular evolution^{3,8}. Second, it suggests that devel-

Evolutionary biology

More mutations in males

Kate Lessells

Mutation is the ultimate source of all genetic novelty and is therefore a key process in evolution. As early as 1947, Haldane¹ suggested that, if copying mistakes during cell replication were a major source of mutations, mutation rates should be higher in males than in females because of the greater number of cell divisions involved in spermatogenesis compared with oogenesis. Writing in *Nature Genetics*, Ellegren and Fridolfsson² confirm Haldane's prediction by analysing the molecular evolution of a pair of genes on the avian sex chromosomes.

Chromosomal sex determination is similar in birds and mammals except that in birds it is the males that have two identical sex chromosomes (ZZ) and the females that are heterogametic (ZW). W chromosomes are passed from one generation to the next only in eggs, whereas Z chromosomes are transmitted one-third of the time in eggs and two-thirds of the time in sperm. So if genes mutate more frequently in males, Z-linked genes should have a higher mutation rate than W-linked genes, and their relative mutation rates will bear a simple mathematical relationship to the relative mutation rates in males and females³. Genes on sex chromosomes can

therefore be used to estimate relative mutation rates in males and females.

Ellegren and Fridolfsson investigated nucleotide-sequence evolution in the *CHD* genes, different versions of which are found on the W (*CHD-W*; refs 4–6) and Z (*CHD-Z*; ref. 7) chromosomes. These genes are the only homologous pair of coding genes that have been identified on the avian sex chromosomes so far. The analysis of homologous pairs of coding genes has two advantages. First, it avoids the possibility that exists for non-homologous sequences of mutation rates differing for reasons other than the sex of the parent³. Second, it allows nucleotide sequences to be unambiguously aligned and sequence differences to be identified³.

By comparing nucleotide sequences from several bird species, the authors estimated the rate of evolutionary change in each of the *CHD* genes. However, nucleotide pairs that affect the proteins encoded by the genes will be subject to selection, and this will confound any attempt to estimate mutation rate from differences in sequences³. Ellegren and Fridolfsson got around this problem by analysing both synonymous nucleotide dif-

opment of the germ line should minimize the number of cell divisions from zygote formation to gametogenesis. So, other things being equal, a monophyletic germ-cell line should split as early as possible from the somatic-cell line — at the first cell division — and the branching tree of cell divisions leading to gametes should be as even as possible, perhaps explaining the existence of rarely dividing 'reserve stem cells'¹¹ among spermatogonia. Third, it implies that the twofold cost of meiosis in females is added to by the extra mutations passed to progeny in sperm, possibly resulting in selection for females that choose young mates with germ lines that have not been ravaged by mutations during cell division¹². □

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Genome sequencing

Panspermia, spores and the *Bacillus subtilis* genome

James A. Hoch and Richard Losick

In a lecture delivered at Harvard University almost three decades ago, Francis Crick declared that he had become disenchanted with the view that life arose on Earth. Instead, he espoused a Theory of Panspermia, which holds that life was sent to Earth from elsewhere in the Universe, and he proposed that the seed of life would have been an extraordinarily resistant spore of the kind produced by *Bacillus subtilis* and related bacteria. In response, Matthew Meselson pointed out that, if this were so, surely the extraterrestrial civilization would have sent a message in the spore. If only mankind was able to sequence DNA, he wistfully conjectured, then we could learn the secret of the Universe from the nucleotide sequence of the contemporary genome of the spore-forming bacterium. Now we have such a sequence, for on page 249 of this issue¹ a worldwide consortium report the 4,214,810-nucleotide sequence of the single chromosome of *B. subtilis*. If the sequence contains a cosmic revelation, it has evidently escaped the scrutiny of the 151 collaborators in this impressive sequencing project. Nonetheless, the *B. subtilis* genome sequence is a milestone in microbiology and of fundamental importance to industry, medicine and basic science.

The complement of genes making up the *B. subtilis* genome contains many surprises. The regulation of gene expression in this organism is known to be quite different from that in *Escherichia coli*. But the finding of genes encoding 18 sigma factors (prokaryotic regulators of gene transcription) is still surprising. It suggests that *B. subtilis* regulates many of its genes in small groups. The expansion of certain gene families (paralogues) is also remarkable, resulting in, for example, 77 different members of the

ABC family of transporter proteins. These transporters are vital pumping systems that use energy from the hydrolysis of ATP to import cell nutrients and signalling molecules into bacteria, and to export toxic by-products of metabolism and noxious agents, such as antibiotics. The large number of ABC transporters in *B. subtilis* indicates that this organism has evolved an elaborate and finely tuned system for chemical communication with its environment. At the other extreme, one-quarter of the genes are present as a single copy and bear no obvious similarity to any other gene discovered so far. Presumably, they play some useful role in *B. subtilis* physiology under conditions that have not yet been mimicked in the laboratory. The presence of several antibiotic-production pathways — including a polyketide pathway occupying two per cent of the genome — indicates that this microorganism can defend its ecological niche. The genes of unknown function, comprising 42% of the genome, are the subject of a worldwide functional analysis programme that will define whether they are expressed and will attempt to assign a function to their products.

One of the valuable legacies that will stem from this work lies in *B. subtilis* being an exemplar for Gram-positive bacteria, a group that includes *Staphylococcus aureus*, *Streptococcus pneumoniae* and the enterococci. Gram-positive pathogens are the leading cause of death from bacterial infections and are the agents of diphtheria, scarlet fever, toxic-shock syndrome, botulism, listeriosis, pneumonia and tuberculosis, to name a few. Many are becoming resistant to well-known antibiotics, and these threaten the very existence of antibiotic therapy, especially in hospitals. The genome sequence of *B. subtilis*



100 YEARS AGO

The so-called "fruit cure", although not much heard of in this country, is well recognised at various places on the continent, where so-called grape-cure stations have been established. ... Many medical authorities in the tenth century become enthusiastic in their writings over the remarkable curative virtues of grapes, whilst a certain Van Swieten, of a more modern date, is said to have "recommended in special cases the eating of twenty pounds of strawberries a day." The same gentleman also reports a case of phthisis healed by strawberries, and cites cases in which maniacs have regained their reason by the exclusive use of cherries as food! These instances rather savour of the miraculous; but there is no doubt the so-called grape-cure, for indigestion and other evils, is carried on in many places on the continent, and that people betake themselves to Meran, Vevey, Bingen, or to Italy and the South of France with the intention of devoting six weeks to the cure, during which time they are expected to have gradually accomplished the feat of consuming from three to eight pounds of grapes daily as the case may be. Grapes are said to exercise a salutary action on the nervous system and to favour the formation of fat, that is to say, when fruit of good quality is employed.

From *Nature* 18 November 1897.

50 YEARS AGO

The rapid immobilization of mammalian spermatozoa which occurs when an ejaculate is diluted with a large volume of physiological saline is the basis of Milovanov's test of 'resistance', used in assessing the value of a specimen for artificial insemination. Milovanov attributes this immobilization to the toxic action of chloride ions. Chang also remarks on the low motility, rate of respiration and fertilizing capacity of rabbit spermatozoa in dilute suspension. Our own observations confirm these findings; rabbit spermatozoa at a concentration of 20 million/ml. in Baker's solution remain motile for two or three days at room temperature, whereas at a concentration of 0.4 million/ml. they are motionless within an hour or two. In our experience this rapid loss of motility is not attributable to a toxic action of chlorides, since chloride-free diluents have an identical action.

From *Nature* 22 November 1947.

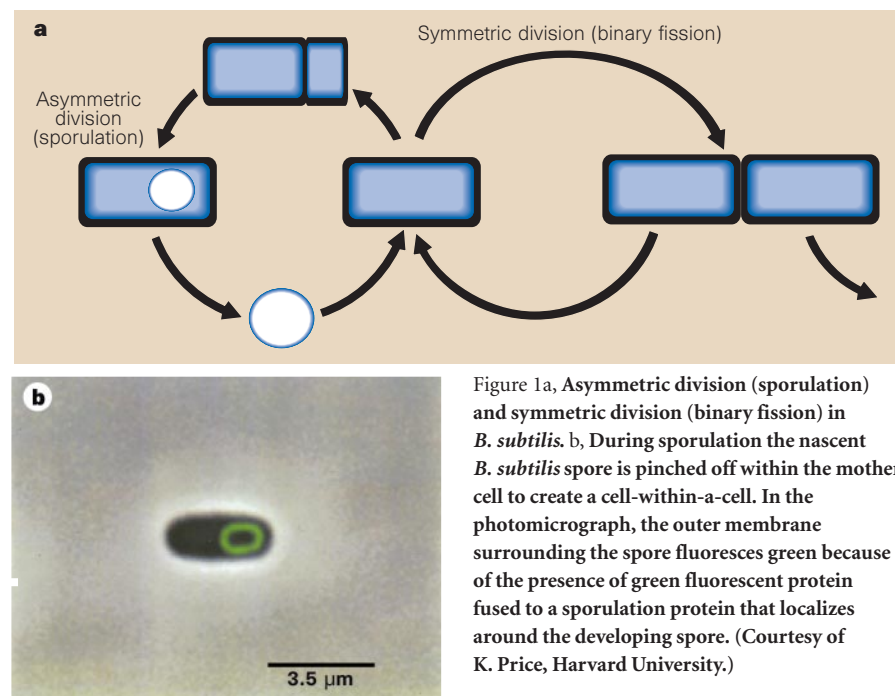


Figure 1a, Asymmetric division (sporulation) and symmetric division (binary fission) in *B. subtilis*. b, During sporulation the nascent *B. subtilis* spore is pinched off within the mother cell to create a cell-within-a-cell. In the photomicrograph, the outer membrane surrounding the spore fluoresces green because of the presence of green fluorescent protein fused to a sporulation protein that localizes around the developing spore. (Courtesy of K. Price, Harvard University.)

provides a solid basis for understanding the genes and genomes of other Gram-positive microorganisms.

Understanding the functions of newly identified genes through their close similarity (homology) to genes of known function is the basis of bioinformatics; the success of this is entirely dependent on how accurately the known genes have been characterized. With the completion of the *E. coli* genome sequence earlier this year², we now have the pre-eminent organisms of both Gram-positive and Gram-negative groups as reference standards for gene identification. Forty years of intensive studies into the genetics, biochemistry and physiology of *B. subtilis* provides a high degree of confidence in such comparisons, especially for Gram-positive microorganisms where *E. coli* information may be of only limited usefulness. Because of the large differences in the cell walls, cell membranes and surface structures of Gram-positive and Gram-negative bacteria, there will be discrete groups of genes unique to each (orthologues) that are likely to hold a rich repository of new targets for antibacterial agents. If the lesson from the *B. subtilis* genome sequence of multiple transport systems is a general phenomenon, then efforts to develop new classes of antibiotics against Gram-positive pathogens should be directed towards the discovery of agents that prevent these bacteria from exporting antibacterial compounds.

A driving force behind genetic, biochemical and cytological studies in *B. subtilis* has been interest in the mechanisms regulating sporulation^{3,4}, a developmental process where a cell undergoes metamorphosis into a dormant spore form that can resist extremes of environment (including, perhaps, those of interstellar travel). Because of

this, we know more about gene expression during the post-exponential phase of growth in *B. subtilis* than in any other bacteria. Yet we are only now beginning to understand how sporulation is linked to DNA replication and the cell cycle. A fundamental question in bacteria, which lack a spindle apparatus, is how they segregate newly duplicated chromosomes. Recent advances in visualizing chromosome movement in *B. subtilis* and other bacteria has prompted a search for the chromosome segregation machinery⁵⁻⁸.

The septation process during cell division also remains a mystery in all bacteria, but particularly in *B. subtilis*, which undergoes symmetric division (binary fission) during growth and asymmetric division during sporulation (Fig. 1a). A hallmark of sporulation is the process of engulfment (phagocytosis) in which the nascent spore is wholly pinched off within the nurturing mother cell to create a cell-within-a-cell (Fig. 1b). Yet little is understood about how the bacterial membranes mediate this remarkable example of prokaryotic phagocytosis. These and other fundamental problems pose challenges in research, the solutions to which will be greatly accelerated by the availability of the entire sequence of the *B. subtilis* genome. □

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Daedalus

Congeaed fat

Our body fat melts at about 17 °C, so that the body can store it in liquid form. Cold-blooded creatures such as fish, and cold parts of warmer animals (such as the feet of cows), contain even lower-melting fats, so as not to risk their freezing in storage. People in cold climates slowly manage to shift the composition of their fat towards lower-melting mixtures, for the same reason. The process is slow, because fat tends to be stored direct from the diet, with whatever melting point it happens to have. Only gradually can it be optimized for the thermal conditions.

Daedalus is now exploiting these facts. He points out that many pure fats melt at well above the 37 °C of our body. Our fat melts at 17 °C only because it is a mixture, with a melting point lower than its components. So he is adding yet another complication to the current obsession with fats in our diet. As well as saturated, unsaturated, polyunsaturated, *cis* or *trans*, omega-3 or omega-6 fats, the dieter can now choose DREADCO's 'oligomolecular' fatty diet. It will contain only one or two specific fats (perhaps tristearin and tripalmitin), and will melt just below core body temperature.

The user's body fat will soon acquire this melting point, with remarkable thermal consequences. Our fat serves us as a heat insulator — which is why it is stored just under the skin. But oligomolecular high-melting fat will also act as thermal ballast. If its owner is exposed to cold, it will start to solidify. It will stay at its high melting point until it has given up all its latent heat of solidification — equivalent to maybe eight hours of the body's entire metabolic output. For all that time, even in the coldest environment, its owner will feel warm as toast. Then the cold will begin to bite seriously, and he will have to warm up again. A hot bath, or better, a bath with sustained heating, could quickly pump back into him the latent heat needed to remelt his frozen fat.

DREADCO's oligomolecular fat diet will transform our thermal resilience. No longer will we need to wrap up warm for short trips outdoors, even in the coldest weather. Traditional icy British bedrooms will lose their terrors; colds and 'flu, often triggered by sudden exposure to freezing conditions, will plummet. And snow clearers, refrigeration engineers, polar-bear hunters and pornographic film stars will be able to enjoy perfect immunity to cold for a whole working shift.

David Jones

Bill's bands

Swiss sculptor Max Bill, working in the middle of this century, created forms aiming to visualize intuitively the mathematics of his day, with their structure determined by a sense of the way space could be 'energized'.

Martin Kemp

The idea that mathematical ideas can exhibit beauty, and more particularly that pure geometrical forms exhibit aesthetic perfection at the highest level, has an ancient ancestry. The figures of the five regular polyhedra — the so-called 'Platonic solids' — together with the sphere were particularly revered for their perfect symmetries, and became favourite objects in Renaissance decorative schemes such as the perspectival panels of inlaid wood that adorned aristocratic studios.

Writing in 1949 (ref. 1), the Swiss sculptor, painter and architect Max Bill (born 1908) delighted in the "aesthetic reaction" provoked by "models at the Musée Poincaré in Paris where conceptions of space have been embodied in plastic shapes or made manifest by coloured diagrams". With the advent of abstraction in classic Modernism in this century, such geometrical forms have been liberated to become self-sufficient subjects for autonomous works of art — and this is just what Bill exploited in his own sculpture.

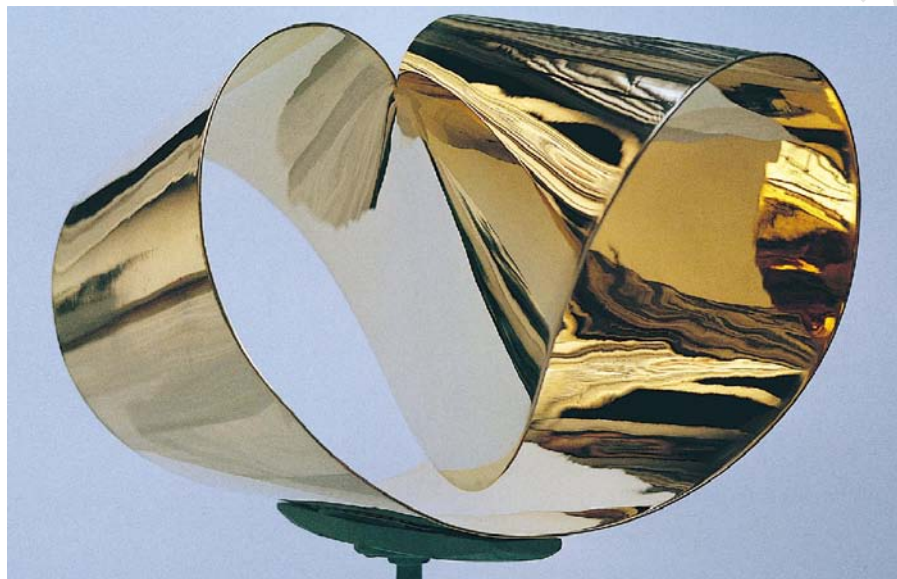
He saw his works as presenting something analogous to the mathematics of his day; as a form of intuitive visualisation of "theorems" that were escaping the reach of any conventional "visualizing agency". He was striving for an art that would be to Einstein what the work of Phidias, the ancient Greek sculptor, was to Archimedes.

One series of sculptures, in a variety of media, works variations of the Möbius band, evoking the idea of a "finite infinity" which Bill characterized as an "essential guide to the speculations of contemporary physicists".

The precise form adopted by Bill's topological variations was determined not by mathematical sense of the way space could be energized to reveal forms that may be active, inchoate, which underlie and every law of physics has the power to fathom".

His aim was not the construction of content based on a sensing of the existence of "fields of force".

Bill was one of a number of artists who were attempting to respond to



Max Bill's *Monoangulated Surface in Space* (gilded brass, 1959).

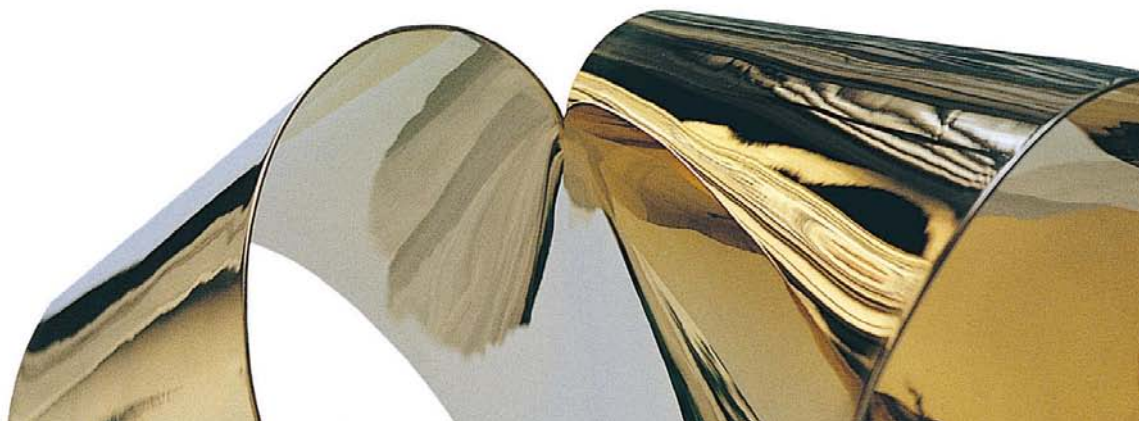
new ideas of space in physics and mathematics. They came face to face with the problem that besets anyone — artist or scientist — who wishes to illustrate four- or multi-dimensional space with the resources available to us for representation in our two- and three-dimensional media. Basically, as I will be showing in a later article, the problem cannot be solved in terms of any model that we can really 'see'. The advantage that the artists possess is that they can exploit suggestion and visual allusion in a way that is not generally acceptable in a scientific or geometrical diagram. Bill felt free to elide geometry, aesthetics and metaphysics without the constraints of analytical accountability that dominate modern science.

To most modern mathematicians, however, much attuned to the aesthetics of spatial

stands very much as an heir to the geometrical cosmographers of the past — not least to Johannes Kepler, the astronomer who in the early seventeenth century first established the elliptical nature of the planetary orbits.

Perhaps the unembarrassed nature of Bill's "astral flights... of the imagination" achieves something that modern science has striven to drive underground, giving the lie to the instinctual passions that motivate and sustain scientific enquiry in its most abstract and mathematically demanding forms. Should we now be condemned to segregate into separate mental zones faculties that past ages could see married in the work of one mind? □

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A prion-linked psychiatric disorder

The finding that new variant Creutzfeldt-Jakob disease, the recently discovered prion disease thought to be associated with ingestion of bovine offal¹, has a prolonged course and can present with psychiatric symptoms² raised the possibility that hereditary forms of prion disease may also be associated with serious psychiatric disorders. We have sequenced the open reading frame of the prion protein (PrP) gene isolated from 10 patients with a strong family history of psychiatric illness. In one patient we discovered a previously undescribed sequence alteration, N171S, which raises the possibility of a significant extension in the pathology associated with inherited prion diseases.

We studied 10 individuals from families with a high incidence of schizophrenic or schizoaffective (DSM-IV) disorders. We sequenced the prion protein gene (*PRNP*) directly from polymerase chain reaction products amplified from peripheral blood leukocytes from each of these patients. In nine families where the disease was confined to schizophrenia we found no mutations, but one other patient exhibited a previously undescribed adenine to guanine substitution, resulting in an asparagine to serine alteration at codon 171 of the *PRNP* gene. The individual was a heterozygote for codon 129, a site of a common polymorphism³, and by subsequent cloning and sequencing we found that the mutated allele encoded valine at codon 129. We also found this genotype in five of eleven living relatives of the patient (Fig. 1).

The patient suffered persecutory delusions, auditory hallucinations, severe depression and had a history of suicide attempts and violent behaviour over a 10-year period. Neurological symptoms such as ataxia or dementia, typical of known prion diseases⁴, were absent. Of the other members of the patient's family identified as mutation carriers, the patient's mother has dementia and urinary incontinence and a 35-year history of similar psychiatric symptoms. The

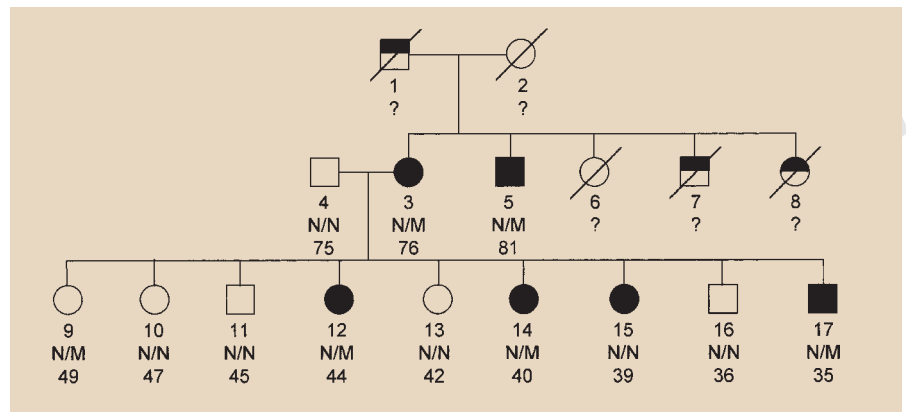


Figure 1 Family pedigree. Squares denote males and circles denote females. Black symbols indicate definitive psychiatric disorders affecting family members and half-black symbols denote the family members with plausible psychiatric symptoms. The age of each individual is shown below the symbols. N, normal; M, mutant allele. The first identified patient was individual number 12.

patient's uncle presented seven years ago with abnormal behaviour including apathy, social withdrawal, mutism and occasional violence, and subsequently developed urinary and faecal incontinence, walking difficulty and dementia. Two affected siblings have a psychiatric history of alternating severe depression and aggressiveness and one of them also has persecutory delusions and auditory hallucinations whereas the third was symptom-free.

This pedigree exhibits incomplete penetrance and variable expression of the disease, typical of inherited prion diseases. None of these patients can be easily classified within well-defined clinical categories and all responded poorly to conventional therapies. Of the five siblings with normal *PRNP* genotype, four have had no psychiatric or neurological illness whereas the fifth has bipolar disorder, characterized by sequences of euphoric, depressive and normal periods. This patient lacks the complex disease progression seen in the family members with N171S.

Our findings indicate that inherited prion

diseases might include serious atypical psychiatric disorders, in addition to the well-defined strictly neurological pathologies⁴ and personality changes⁵. An analysis of a large number of affected families and individuals known to be free of any mental disorders will establish the frequency of the new *PRNP* genotype and the strength of its association with mental disease.

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Trapping speciation

Chromosome rearrangements are often an important factor in the origin of new species¹. They have a potential to affect the fitness of hybrids, and so to stop gene flow and strengthen genetic divergence between intraspecific chromosomal races². Debate still rages over which model¹ best explains chromosomal speciation, the main difficulty being the lack of historical records. We now present data that may represent just such a record.

In the small village of Migiondo (North-

ern Italy) Hauffe and Searle³ found only one of the two chromosomal races of the common house mouse *Mus musculus domesticus* that we had described⁴ 10 years earlier. All over Europe, house mouse populations with the standard karyotype (diploid number $2n=40$ all-telocentric chromosomes) are patchily distributed and parapatrically intermingled with more than 40 other populations. These other populations have reduced chromosome numbers following telocentric fusion and fixation of metacentric chromosomes produced by robertsonian translocation. Robertsonian chromosomes (Rbs) occur at an extremely

high rate, and of the 171 possible telocentric arm combinations that can form Rbs, 124 have been observed. Northern Italy hosts the vast majority of them⁵. Theoretically, each of the chromosomal races may be on the way to speciation^{1,3}.

In the area north of Milan, we and others^{5,6} have been trapping mice for 20 years and have described a population, Milan I, with $2n=24$, containing 8 homozygous pairs of Rbs (arm combinations: 2.4, 3.6, 5.15, 7.8, 9.14, 10.12, 11.13, 16.17; numbers refer to the corresponding telocentric chromosome of the standard mouse karyotype). Last year, during a dioxin risk assessment

survey in the village of Seveso⁷, we captured 23 mice from 3 sites which all had the same karyotype ($2n=24$) and 8 homozygous pairs of Rbs (S. G., M. Z., C. A. R. & E. C., manuscript in preparation). The arm combinations (2.12, 3.4, 5.15, 6.7, 8.11, 9.14, 10.13, 16.17) are different from those expected of the Milan I population, even though Seveso lies within the Milan I distributional area.

The two populations share only three ubiquitous Rbs: 5.15, 9.14 and 16.17. Rb(2.12) and Rb(8.11) have arm combinations that have not previously been described. A comparison with the karyotypes of the surrounding populations⁸ (Milan II, Luino, Lower Valtellina and Cremona) shows that the origin of the Seveso Rbs may be traced parsimoniously from the parapatric $2n=24$ Milan II population (Rbs: 2.8, 3.4, 5.15, 6.7, 9.14, 10.12, 11.13, 16.17) from whole-arm reciprocal translocations (WART) among Rb(2.8), Rb(10.12) and Rb(11.13).

The origin of some of the current Rb populations can be traced back by WARTs among Rbs of a hypothetical ancestral Rb population^{1,5,9} and an actual case of a heterozygous Rb mouse with karyotype derived through WARTs has been described⁹. We suggest that Seveso mice originated over no more than 20 years from the time of massive material movements (during scarification, 1977–1984, and reconstruction, 1985–1994) which favoured contraction and expansion of the Milan I and Milan II territories. Hauffe and Searle³ described a disappearing speciation event in a similarly small village, the surrounding area of which was affected by enormous earth movements (avalanches and floods).

The possible repeated occurrence of appearing and disappearing speciation events could greatly shorten the time needed for new chromosomal races to establish themselves. They could form or disappear within a few decades, a period of time never before suspected. Such events could also better explain the patchy present-day distribution of the house mouse Rb races. The Seveso chromosomal race changes will be worth monitoring.

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Sex ratio unaffected by parental age gap

Sex ratios based on a small sample of births tend to be very unstable. It is therefore not surprising that Manning *et al.* found a relationship between spousal age differences and the sex ratios at birth for a very small sample from a restricted population¹. Their findings do not stand up to scrutiny when tested with larger, representative samples of births.

We examined the sex ratio at birth (males per 100 females) for a pooled sample of 467,693 births to women of reproductive age (ages 15–49) in 12 less-developed countries with a wide range of overall fertility levels. The data come from the Demographic and Health Surveys (DHS), which collected information from nationally representative samples of women in each country between 1992 and 1997. The total fertility rate in these countries ranges from more than six lifetime births per woman in sub-Saharan African countries such as Mali and Zambia to a near-replacement level of fertility of 2.5 births per woman in countries such as Brazil and Kazakhstan. The largest survey, the National Family Health Survey of India, was not formally part of the DHS programme, but used similar questionnaires and survey procedures. Information on births was collected in face-to-face interviews with mothers in the birth-history section of the questionnaire. Because the surveys did not collect information on the ages of previous partners, our analysis is restricted to women who are currently married or living with a man and have been married only once.

Table 1 shows the findings for three different groups: men who are at least five years older than their wives; men who are older than their wives by less than five years; and men who are younger than their wives.

The most significant observation is that sex ratios at birth vary hardly at all by either the birth order or the relative ages of the parents. The overall sex ratio of 105.9 for all births is in the middle of the normal range of 105–107 that has been observed in most parts of the world². Men married to older women are slightly more likely than other men to have male children, but the difference is not large.

Because Manning *et al.* found large differences only for first births, we separated total births into first births ($N=127,176$) and all subsequent births ($N=340,517$). For first births, the differences are even smaller than those observed for total births. Moreover, the slight difference that is evident is in the opposite direction to the pattern found by Manning *et al.* (husbands much older than their wives are slightly less likely to have male children than are other husbands).

We also conducted a similar analysis controlling for age of husband. Again, the analysis failed to confirm the results found in the small sample in England. Finally, we examined the results separately for each of the 12 countries included in the pooled sample. The individual country findings confirm the lack of any consistent pattern of sex-ratio differences in relation to the relative ages of the spouses. In seven countries, the sex ratio at birth was somewhat lower for husbands married to much younger women than for other husbands (contrary to the findings of Manning *et al.*). In the other five countries, the reverse was true.

In conclusion, no matter whom you marry, your chances of having a male child for any given live birth are about 51.4 per cent, which corresponds to a sex ratio at birth of 105.9 males per 100 females.

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J. T. Manning *et al.*¹ categorized the sexes of children by birth order and by difference between parents' ages. There was an excess of first-born sons when the father was 5 years older than the mother, and an excess of first-born daughters when the father was one or more years younger than the mother.

A study³ presented data on 1.4 million single, white, live births in New York State from 1959 to 1967, categorized by maternal age, paternal age and birth order. Maternal and paternal age were categorized into five-

Table 1 Sex ratios at birth (male births per 100 female births)

Birth order	Difference in age of husband and wife			Total
	Wife older than husband	Husband less than 5 years older than wife	Husband at least 5 years older than wife	
First birth	107.5	107.5	106.9	107.2
Second or subsequent birth	106.5	105.6	105.3	105.4
Total	106.7	106.1	105.7	105.9

year age classes (so sex-ratios of offspring of 'toy boys' and 'sugar daddies' are identifiable). For cells with standard errors of less than 0.01, the sex ratios varied within the range 0.51 to 0.53. The sex ratios of offspring of 'toy boys' and 'sugar daddies' do not differ significantly from one another nor from that of the remaining births. Hence the result of Manning *et al.* is specific to the population of Liverpool schoolchildren they studied, and is probably dependent on sampling error.

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I have used the birth records for Illinois from 1989 to 1995 to compare with the report on parental age differences and the sex ratio of first-born children¹. These contain 326,207 single, live first births with no reported anomalies. Couples in which the fathers were at least 5 years older than the mothers had 37,487 first-born sons and 36,173 first-born daughters, a sex ratio of 1.0363. Couples in which the mothers were 1–9 years older than the fathers had 27,816 first-born sons and 26,571 first-born daughters, a sex ratio of 1.0469. This small difference is in the opposite direction to the effect reported by Manning *et al.*, but is well short of statistical significance. Hence the effect that Manning *et al.* reported arises from some factor other than the relative ages of the parents.

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Active uptake of bicarbonate by diatoms

Marine diatoms play a predominant role in the biological carbon pump¹ transferring carbon dioxide from surface to deep waters. Laboratory studies show that a number of species take up HCO_3^- and concentrate inorganic carbon intracellularly allowing rapid growth despite low CO_2 availability^{2,3}. In contrast, many oceanographers, particularly when interpreting carbon isotope data^{4,5}, have made the assumption that diatoms do not utilize the abundant HCO_3^- in seawater but rather take up CO_2 by diffusion⁶. This has led to the hypothesis that large diatoms may be CO_2 -limited in the oceans⁷. We now demonstrate active uptake of HCO_3^- in the field and a carbon-concentrating mechanism in coastal Atlantic diatoms. By manipulating p_{CO_2} we show that growth of large diatoms in the California upwelling is not

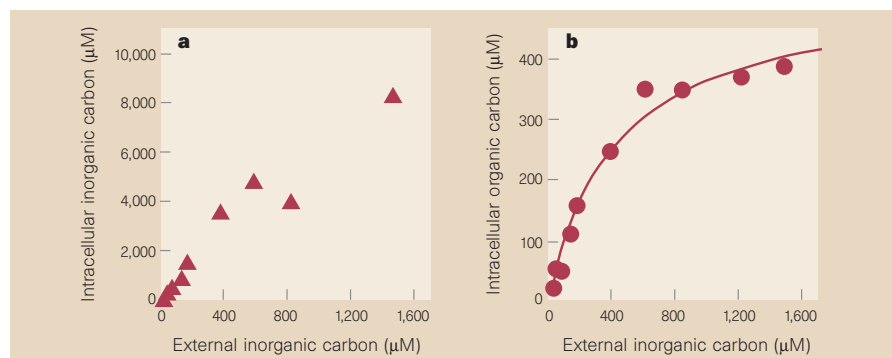


Figure 1 Short-term inorganic carbon uptake (a) and photosynthesis (b) in a marine diatom assemblage. Phytoplankton collected from Delaware Bay in April 1997 were filtered onto 5 μm polycarbonate filters and resuspended in low-carbon, buffered sea water, pH 8.2. Cell suspensions illuminated in an O_2 electrode chamber were allowed to deplete residual DIC until they reached the CO_2 compensation point (photosynthesis=respiration). Cell suspensions (190 μl) were incubated with varying concentrations of $\text{H}^{14}\text{CO}_3^-$ (50 mCi mmol^{-1}) for 10 s. Cells were centrifuged through a layer of silicone oil into a basic terminating solution (2.5 M NaOH, 10% methanol)⁸ and frozen cell pellets were assayed for organic (acid-stable) and inorganic (acid-labile) ^{14}C activity by liquid scintillation counting. Total carbon counts were normalized to biovolume in cell pellets measured by incubating cell suspensions for 1 min with $^3\text{H}_2\text{O}$ (ref. 8).

limited by CO_2 availability.

We ran short-term $\text{H}^{14}\text{CO}_3^-$ -uptake experiments⁸ using samples dominated by large ($>30 \mu\text{m}$) diatoms (*Asterionella*, *Nitzschia* and *Rhizosolenia*) collected from Delaware Bay in 1997 during a spring phytoplankton bloom. The phytoplankton rapidly took up $\text{H}^{14}\text{CO}_3^-$ (within 10 seconds at pH 8.2) and accumulated intracellular dissolved inorganic carbon (DIC) at concentrations roughly 10 times higher than those outside (Fig. 1a). This resulted in high rates of photosynthesis at low ambient DIC levels.

The apparent cellular half-saturation constant (K_m) for photosynthesis was $\sim 2.5 \mu\text{M}$ CO_2 (Fig. 1b), consistent with those measured in laboratory cultures of marine diatoms³. We have recently obtained similar results from 10-second uptake experiments in the California upwelling where the indigenous diatoms accumulated cellular inorganic carbon pools five times higher than external levels and showed photosynthetic half-saturation at $\sim 2 \mu\text{M}$ CO_2 .

The rapid ^{14}C uptake by the Delaware Bay diatom assemblage may have occurred through active transport of HCO_3^- or by extracellular catalysis of HCO_3^- dehydration to CO_2 coupled to active CO_2 transport⁹. The addition of 50 μM ethoxy-zolamide, an inhibitor of carbonic anhydrase, the zinc enzyme that catalyses HCO_3^- dehydration, had no effect on cellular DIC accumulation but did decrease carbon fixation to 30% of the control level. Carbonic anhydrase is therefore required to catalyse intracellular dehydration of actively imported HCO_3^- , thereby supplying CO_2 to the carboxylating enzyme Rubisco. Indeed, carbonic anhydrase activity seems to be essential for efficient photosynthesis in laboratory diatom cultures, particularly under low CO_2 conditions^{2,10}. In zinc-limited cultures, the activity of carbonic anhydrase is greatly reduced and

HCO_3^- utilization is impaired, resulting in CO_2 limitation¹⁰.

Diatoms that can use HCO_3^- as a source of inorganic carbon should not be dependent on free CO_2 for growth¹⁰. We tested this hypothesis using samples collected from the coastal California upwelling in June 1996 which we incubated with bubbled air containing CO_2 at various partial pressures. The growth of the phytoplankton, which was dominated by large diatoms (*Thalassiosira* spp., 30–40 μm diameter), was independent of p_{CO_2} (Fig. 2). Further, steady-state growth rates of the cells at 100 p.p.m. CO_2 (~ 1.4 cell divisions per day) were significantly larger than the maximum possible rates that could

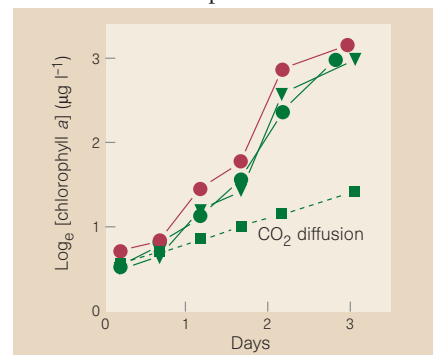


Figure 2 Growth of a Monterey Bay diatom assemblage at various p_{CO_2} levels. Surface water samples (20 litres), collected in June 1996, were incubated with trace-metal-clean techniques at ambient sea temperature and bubbled with air/ CO_2 mixtures containing 100 (green circles), 330 (purple circles), and 750 (green triangles) p.p.m. CO_2 . Growth was measured by daily chlorophyll *a* concentrations¹². Phytoplankton growth rates ($\sim 1 \text{ d}^{-1}$) were not significantly different between CO_2 treatments ($P>0.05$). Dotted line: theoretical maximum growth rate that could be supported by CO_2 diffusion to the cell surface (calculated for a cell radius of 15 μm , 300 pmol C per cell; 3 μM CO_2 in the bulk medium and no CO_2 at the cell surface⁷).

be supported by CO₂ diffusion to the cell surface⁷ (~0.3 divisions per day; Fig. 2). This provides compelling evidence that HCO₃⁻ is an important source of inorganic carbon for the natural diatom population.

Bloom-forming diatoms use HCO₃⁻ as a source of DIC, they possess a carbon concentrating mechanism, and as a result, are not limited by CO₂ availability. If this is the case in productive coastal waters it is probably also the case in the open ocean where higher ratios of DIC to nitrate and phosphate, lower maximum pH during the growth season, and smaller phytoplankton size should make the acquisition of inorganic carbon easier. It is doubtful that inorganic carbon is limiting in the open ocean, unless some other factor, such as low zinc availability¹¹, impairs its uptake and fixation by phytoplankton¹⁰. Our results have important implications for ocean carbon cycling, and suggest that a critical evaluation of carbon isotope fractionation models^{5,6} which assume passive CO₂ uptake by phytoplankton is needed.

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An atom-focusing mirror

The recent interest in atom-optics has mainly been directed at the manipulation of atomic beams by static fields or lasers^{1–3}. Using an alternative approach we have succeeded in focusing in two dimensions a neutral atomic helium beam at room temperature with a reflective optical element (an atom mirror). Such focusing relies on specular elastic scattering, which leaves the coherence of incoming wavepackets unchanged.

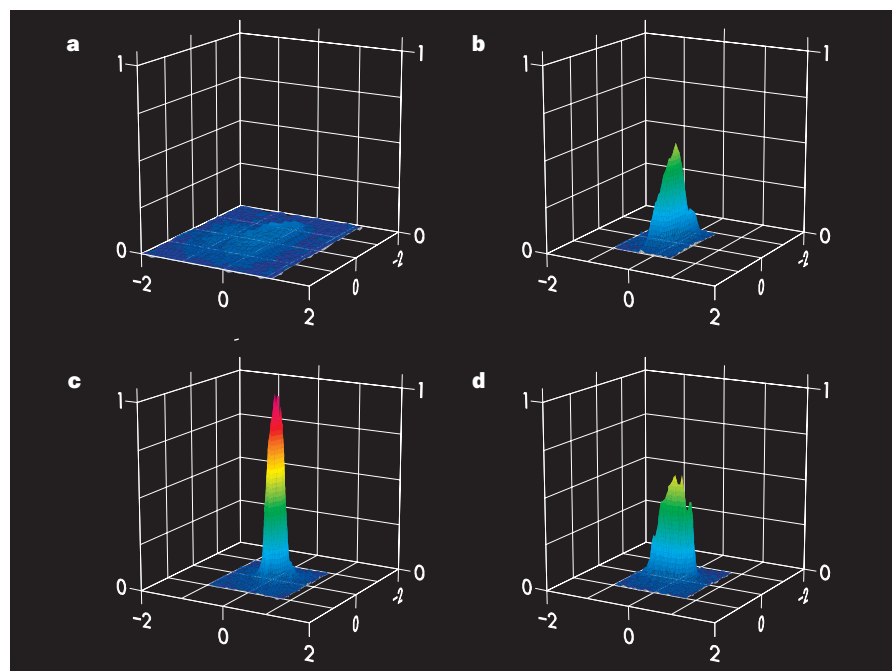


Figure 1 Beam cross-sections for various mirror curvatures (R). The horizontal axes span a plane perpendicular to the beam direction of travel (scale in mm). The vertical axis shows normalized intensity. **a**, Unfocused beam. **b**, First focus in scattering plane ($R=1.2$ m). **c**, Disk of least confusion ($R=0.8$ m). **d**, Second focus perpendicular to scattering plane ($R=0.5$ m).

We created the atom mirror from a single 50- μm -thick silicon crystal cut along the (111) crystal plane. The surface was hydrogen-passivated *ex-situ*⁴ making it inert (the reflectivity remained constant over several months at 10^{-6} mbar). The crystal was deformed electrostatically in an arrangement similar to a parallel plate capacitor to give a parabolic profile for focusing. The focal length can be varied *in situ* simply by adjusting the electric field.

We produced a helium beam in a supersonic expansion source⁵ at room temperature (corresponding to a wavelength of 0.52 Å). We placed the mirror 0.7 m from the source and 0.8 m from the detector with the beam incident at 45°. We measured beam cross-sections for different mirror curvatures (Fig. 1) by scanning the beam across a 100 μm pinhole in front of the detector. Focusing in the geometry used here is necessarily astigmatic. The intermediate disk of least confusion (Fig. 1c) has a spot diameter of 210 ± 50 μm . The solid angle of the unfocused beam is reduced by a factor of about 100. There is a corresponding increase in intensity. The spot size is not limited by aberrations of the mirror, but solely by the geometry of the system and the size of the object (confirmed by computer simulations to be the surface of last scattering in the supersonic expansion⁵).

The ultimate performance of the mirror is limited by the geometrical aberrations⁶ and diffraction⁷ due to the finite size of the mirror (elastic scattering gives no chromatic aberrations). The only other atom-focusing method that relies solely on the de Broglie

wavelength of the atoms is a Fresnel zone plate⁸. A zone plate is diffraction-limited because of the necessary constraints on the plate diameter (about 0.2 mm with current technology). A mirror, on the other hand, has no such inherent limit on its size. With normal incidence, a point source, and image planes 1 m and 0.1 m from the focusing element, the best spot size would be about 2.5 nm with the beam spanning about 2 mm on the mirror surface.

It follows that a helium microscope with nanometre resolution is possible. A helium atom microscope would be a unique non-destructive tool for reflection or transmission microscopy. It could be used to investigate fragile and insulating materials such as polymers and certain biological samples. Focusing mirrors also have the potential to increase spatial resolution and intensity in conventional helium-surface scattering instruments.

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When the Cold War reached boiling point

The Kennedy Tapes: Inside the White House During the Cuban Missile Crisis

edited by Ernest R. May and Philip D. Zelikow
Harvard University Press: 1997. Pp. 728. \$35, £23.50

'One Hell of a Gamble': Khrushchev, Castro, Kennedy, 1958–1964

by Aleksandr Fursenko and Timothy Naftali
Norton/John Murray: 1997. Pp. 420. \$27.50, £25

David Holloway

More than 30 years after the event, the Cuban missile crisis of October 1962 continues to exercise a deep fascination. It brought the world to the brink of destruction, and seared itself into the consciousness of all who lived through it. It is not surprising therefore that it is the most intensively studied of all the Cold War crises.

Over the years, memoirs and declassified documents have provided new evidence. Now Ernest R. May and Philip D. Zelikow of Harvard University have made available the transcripts of recordings of pivotal White House deliberations during the crisis. In the summer of 1962, President John F. Kennedy had installed a recording system in the Cabinet Room and the Oval Office, and he used this system, whose existence was known to only one or two people apart from himself, to record the meetings of the committee of advisers who helped him to formulate his response to the Soviet deployment of medium-range missiles in Cuba.

May and Zelikow deserve great credit for transcribing these tapes. The sound quality of the recordings is abysmal, and a team of court reporters was brought in to prepare the first draft. Not everything could be recovered, and "[unclear]" appears often in the text. But the result is an extraordinary document. Historians will pore over it with great interest, and the general reader will find here a riveting account of the most dangerous crisis of the Cold War. May and Zelikow provide annotations to help the reader to follow the course of events, and an introduction and conclusion to set the crisis in context. Though they comment on the new material, they do not attempt a definitive new interpretation.

The transcripts cover the period from 16 October, when Kennedy received intelligence that the Soviet Union was deploying medium-range missiles in Cuba, to 29 October, the day after he learned of Nikita Khrushchev's agreement to withdraw the missiles. Much of the substance of the White House discussions was already known from the memoirs of participants. The discovery of the missiles in Cuba provoked a debate



Friends and brothers: above, Castro and Khrushchev; below, Robert (left) and John F. Kennedy.

between those who favoured an air strike against the missiles and those who advocated the imposition of a blockade on Cuba. Even after imposing the blockade, Kennedy faced the problem of getting Khrushchev to remove the missiles. Here too opinions were divided. Discussion of possible political means of achieving this goal took place against the backdrop of preparations for an air strike and an invasion of Cuba.

The tapes do nonetheless throw a great deal of new light on the formation of US policy. Berlin, for example, played a more important role in the crisis than later accounts have allowed. Kennedy and his advisers assumed that Berlin was Khrushchev's main goal. Khrushchev had been threatening for four years to sign a separate peace treaty with East Germany. The United States had opposed this, because it would call into question the Western position in West Berlin. Kennedy had made it clear that the United States would be willing to use nuclear weapons in response to any Soviet attempt to occupy West Berlin by force.



When the United States discovered the missiles in Cuba, Kennedy assumed that Khrushchev was intending to announce the existence of the missiles on a visit he was due to make to the United Nations in November. He was afraid that Khrushchev would calculate that, once Soviet missiles were installed in Cuba, the United States would be less willing to use nuclear weapons in Central Europe, where the Soviet Union had superior conventional forces. He feared that the credibility of the US commitment to West Berlin would thereby be undermined. This was one reason why it was important to have the missiles removed from Cuba, but this assessment also weighed against an invasion because that would entangle US forces in an area of secondary importance.

The tapes throw revealing light on Kennedy's leadership. He did not speak at length at the meetings, but it is clear he listened intently. He kept the meetings on the main issues without foreclosing discussion. He was acutely aware that the responsibility for decisions rested on his shoulders. After the initial shock of discovery, he saw more consistently than others the need for a political solution. In the second week, after he had imposed the blockade, he became interested in the possibility of withdrawing US Jupiter missiles from Turkey in return for the removal of the Soviet missiles from Cuba. "We're either going to trade them out, or we're going to have to go in and get them out ourselves," he told his advisers.

On 26 and 27 October Kennedy and his advisers discussed the intricacies of such a trade. They were afraid that it would anger the Turks and undermine NATO (North Atlantic Treaty Organization) by weakening the credibility of US security guarantees.

On the evening of 27 October Robert Kennedy, the Attorney General, informed the Soviet ambassador, Anatoly Dobrynin, that the United States would remove the

Jupiter missiles within a matter of months. Robert Kennedy insisted to Dobrynin that this assurance should remain secret and that, if the Soviet Union made it public, the assurance would be withdrawn. President Kennedy was thus able to earn a reputation for toughness while showing flexibility and a willingness to compromise. The agreement on the Jupiters remained secret for many years — although the missiles were removed in 1963.

President Kennedy did not believe that Khrushchev wanted war, any more than he did himself. He believed that Khrushchev wanted to redress the nuclear balance to put pressure on the Western powers in Berlin. In recent years the most common interpretation has been that Khrushchev wanted to defend Cuba against a US attack.

Aleksandr Fursenko and Timothy Naftali do not resolve this issue in their book. The way they set up their account gives pride of place to the defence of Cuba, but they also provide evidence to show Khrushchev's concern about the way in which the Soviet delay in deploying strategic missiles hampered his foreign policy.

Fursenko and Naftali bring new evidence from Russian archives to bear on the crisis. They show that relations between the Soviet Union and Castro's regime were close and complex before the crisis. They argue that Khrushchev was in charge of Soviet policy during this period, and that whatever was erratic about Soviet policy sprang not from political struggles inside the Kremlin but from Khrushchev's own personality.

They also indicate that Robert Kennedy's conversation with Dobrynin about the Jupiters was not decisive in resolving the crisis, because Khrushchev had already decided to agree to withdrawal of the Soviet missiles from Cuba before he learned of Kennedy's assurance that the Jupiters would be withdrawn from Turkey.

Although they provide a great deal of new information, Fursenko and Naftali do not say enough about the sources they have used. They have been able to consult documents in the Russian Presidential Archive, to which access is very restricted. But they do not reveal what collections they could consult. They quote few documents at length, even though they evidently used minutes of Politburo meetings. One reason why these questions matter is that if the authors had access only to collections dealing specifically with Cuba, that would lead to an emphasis on the defence of Cuba as a motive for Khrushchev's actions. Interesting though the new Soviet sources are, they do not compare in quality with the Kennedy tapes. This suggests that we still have more to learn about the Cuban missile crisis. □

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Ape people

Next of Kin: What Chimpanzees Have Taught Me About Who We Are

by Roger Fouts with Stephen Tukul Mills
Morrow: 1997. Pp. 420. \$25

Marc D. Hauser

The philosopher Ludwig Wittgenstein argued that even if lions could speak we wouldn't understand them. The psychologist David Premack mused that even if chickens had syntax, they'd have nothing interesting to say. But what if both these arguments are wrong, leaving the possibility that, like the fictitious veterinarian Dr Doolittle, we can talk to animals and learn interesting things about their lives? And if we could, whose lives would be worth learning about? If you were a psychologist interested in the human mind and its evolutionary history, you would, without doubt, be driven towards our closest living relative: the chimpanzee.

Roger Fouts is a psychologist. *Next of Kin*, written with Stephen Tukul Mills, is Fouts's personal account of what it is like to work with, talk to and defend the lives and rights of captive chimpanzees. It is an adventure story rife with sadness and horror, excitement and intrigue. It begins with his childhood experiences with animals, his dreams of becoming a child psychologist and, finally, his involvement as a graduate in experimental psychol-

ogy with the ground-breaking research of Allen and Beatrix Gardner and their newly acquired chimpanzee, Washoe.

The Gardners began a research programme designed to reveal the linguistic potential of chimpanzees, specifically the ability to learn words in American sign language (ASL) and to string them together into novel, syntactically structured sentences. Fouts's apprenticeship involved caring for Washoe, treating her like a human child — albeit a deaf child who needed to learn ASL rather than spoken language. The job turned into a life-long passion and led to a fervent interest in the care and well-being of captive animals.

In the late 1960s and early 1970s, several research programmes sparked a renewed interest in human nature and the attributes that had long been touted as uniquely human. Jane Goodall's studies of chimpanzees in the Gombe Reserve were beginning to mature, uncovering evidence of tool use, hunting, murder and social politics. Peter Marler and others working on the natural communication of animals were uncovering exciting parallels to human speech and language. And a group of psychologists, including the Gardners, were attempting to determine whether apes and dolphins could acquire human-like language and demonstrate human-like thought.

In contrast to studies in the wild, work with these animals took on a cult-like fol-

Is this the origin of art?

Is art a human invention or an instinct shared with other animals? The work of primate painters this century has been analysed, debated, shown in galleries and sold for high prices. Joni the chimpanzee, seen here with the Russian scientist Nadjeta Kohts in 1913, was the first to be seriously studied. A later chimp called Washoe (see the review above) apparently claimed in sign language that she liked red best because it was beautiful. Is this all just creative play? The ethologist Desmond Morris, who contributes a foreword to *Monkey Painting* by Thierry Lenain (Reaktion Books, £14.95/\$24.95) believes that apes are expressing a primitive aesthetic sense. Read this intriguing account, look at the pictures and decide for yourself.



lowing, with ape fan clubs, newsletters, prime-time television spots and so on. Many of the researchers invested years of time and energy — as well as emotional attachment — in their studies. Indeed, their subjects were sometimes treated not so much as targets of scientific investigation but rather as family members. It is this paradox that lies at the heart of Fouts's story.

At the start of *Next of Kin*, Fouts mentions the ethical problem of raising a human child in a chimpanzee family. But then, as if the ethical concerns evaporate, he swiftly turns to the fascination of raising a chimpanzee in a human family, and his own childhood "experiments" with cross-fostering ducklings to domestic cats. After spending years experimenting with Washoe and several other chimpanzees, though, he suddenly realizes he has done something unethical. Driven only by his intellectual curiosity, he has failed to consider what such experiences would be like for a chimpanzee held captive without maternal nurture, sex, play, social politics and so on. Fouts retreats from science and plunges into alcoholism.

He eventually emerges from his depression with a renewed sense of energy, guided by a desire to uncover, and ultimately abolish, what he perceives as an international disaster: highly inappropriate housing facilities for primates. The rest of the book tells of Fouts's fight for the care and well-being of primates in captivity, of his continued struggle with research and of the findings that emerged from his studies.

Sociopolitical saga apart, there are various scientific nuggets dispersed throughout the book. The reader is treated to observations of chimpanzees teaching their young bits of ASL, deceiving Fouts for a Coke, signing about the grief of losing a baby, using mirrors to put on make-up and clothes, and signing among themselves about the day's events. One cannot help but be amazed by all of this. Yet Fouts is out of touch with several areas he discusses, and apparently forgets that his research rests on that of others. For example, we find no mention of Premack's work with chimpanzees. Uniquely for his time, Premack focused on the conceptual abilities of animals rather than their capacity to acquire the formal structure of language; it was in this guise that Premack mocked the imaginary syntax-carrying chicken.

And, as it turns out, this is precisely where work on captive apes and other animals has moved, due in part to the critical blows dealt by linguists, psycholinguists and philosophers to studies of ape semantics and syntax. Tetsuro Matsuzawa is also not mentioned and Sue Savage-Rumbaugh, one of the leaders in this field, receives a one-sentence nod. And yet, like Premack, both have generated fantastic findings about the conceptual abilities of chimpanzees and bonobos.

Next of Kin is also replete with conceptual

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Bright Paradise: Victorian Scientific Travellers

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"Extremely readable", D. E. Allen, *Nature* 383, 491 (1996).

and empirical inaccuracies. Fouts thinks that human language is primarily a cultural phenomenon. Given all the evidence of dedicated linguistic brain areas, universal grammar, highly constrained sequences of language development and so on, one simply cannot ignore the importance of biology. He argues in favour of human languages emerging from a gestural form, a claim soundly rejected by many authors, including Steven Pinker, Derek Bickerton and Philip Lieberman. If gestural languages were dominant, why don't we see any remnants today? Why has no culture ever taken a gestural form of language as its first and dominant form of communication? Why aren't gestural signals dominant to vocal signals in most non-human primates?

Fouts claims that the dominant form of communication for human infants is through facial expressions and hand gestures, rather than vocal signals, because human infants are born with a chimpanzee-like vocal tract that does not become fully adult-like until about two to three years of age, when words are strung together into sentences. None of this is correct. Infants cry from birth and soon after begin to make communicative gurgles, raspberries and laughing sounds. Babbling emerges at just about the age that the larynx descends into

the throat, forming the adult-like configuration. The visual and auditory modalities are used for communication in early development. When words are strung into sentences, it is not because of a mature vocal tract, but because of a computational mechanism sufficiently developed to allow recombination.

Further, Fouts's view that all primate vocalizations (that is, those produced naturally as part of the species-typical repertoire) are processed by the emotional areas of the brain is also incorrect, as demonstrated by recent neurophysiological studies on rhesus macaques showing involvement of the auditory cortex.

The book is therefore both fascinating and infuriating. Fascinating because it tells of the trials and tribulations of trying to communicate with another species. Infuriating because the distinction between evidence and impression are blurred, and because profound philosophical and psychological problems are often dealt with superficially. In the acknowledgments, Fouts showers "thank-yous, hugs, and pant-hoots ... to the five people who inspired [the] book: Washoe, Loulis, Moja, Tatu, and Dar". People? How interesting. □

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A fan writes

NASA/TREK: Popular Science and Sex in America

by Constance Penley
Verso: 1997. Pp. 169. \$64.95, £35 (hbk); \$14, £11 (pbk)

Oliver Morton

Just before writing this review I was looking for a US Air Force document on the World Wide Web. It turned out to be on a server called Tuvok — the name of the Vulcan security officer on the eponymous Starship Voyager in the fourth of the *Star Trek* television shows. From seeing this name, I learnt a little about the self-image and style of this corner of the military industrial complex. I also recognized again the force of Constance Penley's case in *NASA/TREK*: that *Star Trek* is everywhere, and that the way people make use of it can reveal a lot about science, technology and — her particular interest — sex.

As Penley argues, "going into space" is the governing metaphor by which people understand the world of science and technology, not to mention their place in that world as women and men. And *Star Trek* is the dominant vision of space travel in today's culture.

Penley looks at the way this body of modern myth has been appropriated by two very different groups, one mostly of white male engineers, many of them in America's South, the other of heterosexual women with "pink-collar, 'subprofessional' and high-tech service industry" jobs, many in California. The first group — the National Aeronautics and Space Administration (NASA) — has attempted to lay claim to the television pro-

gramme's Utopian vision of spaceflight. The second group — the "slashers" — has entertained itself producing and distributing pornographic romances centred on a physical relationship between Captain Kirk and Mr Spock. This is the world of "K/S" fiction: the slashers take their name from the punctuation between their subjects' initials.

Unsurprisingly, NASA and the rest of the US aerospace establishment are more than willing to link themselves to *Star Trek*, both out of genuine affection and as good public relations. There has been a *Star Trek* exhibition at the Smithsonian National Air and Space Museum; the first (non-orbital) space shuttle was called the Enterprise; the planned replacement for the Hubble is known as Space Telescope Next Generation; the first black female astronaut prefaced her transmissions with Lieutenant Uhura's catchphrase "Hailing frequencies open".

Penley sees NASA as attempting to define its place in popular culture as the practical realization of *Star Trek*'s Utopian 'theory' of space travel. She herself sees the relationship between the two 'narratives' as a little deeper than that; like the slash in K/S, the slash in *NASA/TREK* stands for a more intimate and subversive reading of a relationship normally taken at face value.

Most of the first half of Penley's book is devoted to the Challenger disaster and the death of schoolteacher Christa McAuliffe, with whose carefully chosen mediocrity television-watching America was supposed to identify. (Pam Dawber, star of the alien-around-the-house sitcom *Mork and Mindy*, was presumably given her place on the selection panel to spot someone with the requisite girl-next-dooriness.) From tasteless jokes onwards, Penley analyses the effects of the disaster on NASA's attempts to write itself as popular culture, and fits these into the history of women's treatment in the space programme.

In the second part of the book, she turns her attention to the slashers and finds that they have rewritten the *Trek* myth to their own ends far more successfully. By choosing to "work with what's out there", the slashers have created a literature about sex and Utopia that does not repress the body or subjugate women and that cuts across race (indeed species) barriers. The women who create these fictions go from being passive viewers to active creators: they confess themselves much more embarrassed to be known as television fans than as pornographers.

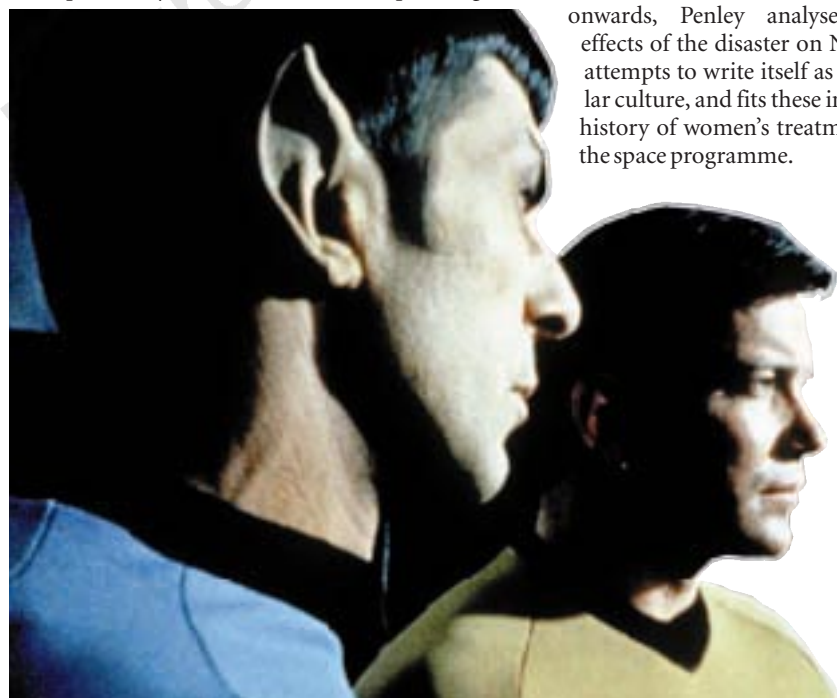
As is often the way with cultural studies, *NASA/TREK* succeeds not so much by force of argument as by the accumulation of telling details and resemblances. Penley provides plenty of them and teases out their relationships provocatively. Although there are places where one would like a little more reporting — how did the quasi-military world of *NASA/TREK* respond to the gays in the military debate? — the book succeeds most admirably in its own terms.

It also does more. It reveals a way of talking about science that is widely ignored and utterly undervalued: that of the fan. People who are not fans find it hard to understand the nature of the fan's relationship to his or her field, be it Arsenal football club, the Grateful Dead, *Star Trek* or whatever. The fans are experts, they are enthusiasts, they are critics and they are creators, writing stories, publishing 'zines and remixing bootleg tapes. They know the details and they delight in spotting the mistakes, but at the same time they imbue with value and significance whatever it is they're attached to. They are a community of consumption convinced it is central to the whole process of production.

The sciences have boosters, enthusiasts, amateur practitioners and — I fear — even worshippers. But only rarely do they have the sort of organized fandom common to sports and genre fiction. There are exceptions: it is not to belittle them to say that some AIDS activists share the attributes of fans, attacking science when it fails them but valuing it highly at the same time. But, in general, the idea of a community of non-practitioner enthusiasts, fiercely critical when the rules are broken and convinced that they are central to the process, is alien to most of science, and that is a great pity.

The fan's dual nature as insider and outsider provides a voice that could be valuable in areas where the social implications of science matter, a voice that can promote debate without entrenching hostility. Much of the power of Penley's book is that she writes as a fan of both *NASA* and *Star Trek*, with a vivid appreciation of what fun fans can have and what good they can do. More such fans are urgently needed elsewhere. □

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"Those pink-collar women think we're lovers. Surely that's illogical, Captain ..."

The complete genome sequence of the Gram-positive bacterium *Bacillus subtilis*

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***Bacillus subtilis* is the best-characterized member of the Gram-positive bacteria. Its genome of 4,214,810 base pairs comprises 4,100 protein-coding genes. Of these protein-coding genes, 53% are represented once, while a quarter of the genome corresponds to several gene families that have been greatly expanded by gene duplication, the largest family containing 77 putative ATP-binding transport proteins. In addition, a large proportion of the genetic capacity is devoted to the utilization of a variety of carbon sources, including many plant-derived molecules. The identification of five signal peptidase genes, as well as several genes for components of the secretion apparatus, is important given the capacity of *Bacillus* strains to secrete large amounts of industrially important enzymes. Many of the genes are involved in the synthesis of secondary metabolites, including antibiotics, that are more typically associated with *Streptomyces* species. The genome contains at least ten prophages or remnants of prophages, indicating that bacteriophage infection has played an important evolutionary role in horizontal gene transfer, in particular in the propagation of bacterial pathogenesis.**

Techniques for large-scale DNA sequencing have brought about a revolution in our perception of genomes. Together with our understanding of intermediary metabolism, it is now realistic to envisage a time when it should be possible to provide an extensive chemical definition of many living organisms. During the past couple of years, the genome sequences of *Haemophilus influenzae*, *Mycoplasma genitalium*, *Synechocystis* PCC6803, *Methanococcus jannaschii*, *M. pneumoniae*, *Escherichia coli*, *Helicobacter pylori*, *Archaeoglobus fulgidus* and the yeast *Saccharomyces cerevisiae* have been published in their entirety¹⁻⁸, and at least 40 prokaryotic genomes are currently being sequenced. Regularly updated lists of genome sequencing projects are available at <http://www.mcs.anl.gov/home/gaasterl/genomes.html> (Argonne National Laboratory, Illinois, USA) and <http://www.tigr.org> (TIGR, Rockville, Maryland, USA).

The list of sequenced microorganisms does not currently include a paradigm for Gram-positive bacteria, which are known to be important for the environment, medicine and industry. *Bacillus subtilis* has been chosen to fill this gap^{9,10} as its biochemistry, physiology and genetics have been studied intensely for more than 40 years. *B. subtilis* is an aerobic, endospore-forming, rod-shaped bacterium commonly found in soil, water sources and in association with plants. *B. subtilis* and its close relatives are an important source of industrial enzymes (such as amylases and proteases), and much of the commercial interest in these bacteria arises from their capacity to secrete these enzymes at gram per litre concentrations. It has therefore been used for the study of protein secretion and for development as a host for the production of heterologous proteins¹¹. *B. subtilis* (*natto*) is also used in the production of Natto, a traditional Japanese dish of fermented soya beans.

Under conditions of nutritional starvation, *B. subtilis* stops growing and initiates responses to restore growth by increasing metabolic diversity. These responses include the induction of motility and chemotaxis, and the production of macromolecular hydrolases (proteases and carbohydrases) and antibiotics. If these responses fail to re-establish growth, the cells are induced to form chemically, irradiation- and desiccation-resistant endospores. Sporulation involves a perturbation of the normal cell cycle and the differentiation of a binucleate cell into two cell types. The division of the cell into a smaller forespore and a larger mother cell, each with an entire copy of the chromosome, is the first morphological indication of sporulation. The former is engulfed by the latter and differential expression of their respective genomes, coupled to a complex network of interconnected regulatory path-

ways and developmental checkpoints, culminates in the programmed death and lysis of the mother cell and release of the mature spore¹². In an alternative developmental process, *B. subtilis* is also able to differentiate into a physiological state, the competent state, that allows it to undergo genetic transformation¹³.

General features of the DNA sequence

Analysis at the replicon level. The *B. subtilis* chromosome has 4,214,810 base pairs (bp), with the origin of replication coinciding with the base numbering start point¹⁴, and the terminus at about 2,017 kilobases (kb)¹⁵. The average G + C ratio is 43.5%, but it varies considerably throughout the chromosome. This average is also different if one considers the nucleotide content of coding sequences, for which G and A (24% and 30%) are relatively more abundant than their counterparts C and T (20% and 26%). A significant inversion of the relative G - C/G + C ratio is visible at the origin of replication, indicating asymmetry of the nucleotide composition between the replication leading strand and the lagging strand¹⁶. Several A + T-rich islands are likely to reveal the signature of bacteriophage lysogens or other inserted elements (Fig. 1, see below).

We have analysed the abundance of oligonucleotides ('words') in the genome in various ways: absolute number of words in the genomic text, or comparison with the expected count derived from several models of the chromosome (for example, Markov models, or simulated sequences in which previously known features of the genome were conserved¹⁷). Comparing the experimental data with various models allowed us to define under- and overrepresentation of words in the experimental data set by reference to the model chosen. In general, the dinucleotide bias follows closely what has been described for other prokaryotes^{18,19}, in that the dinucleotides most overrepresented are AA, TT and GC, whereas those less represented are TA, AC and GT. Plots of the frequencies of AG, GA, CT and TC in sliding windows along the chromosome show dramatic decreases or increases around the origin and terminus of replication (data not shown). Trinucleotide frequency, directly related to the coding frame, will be discussed below. The distribution of words of four, five and six nucleotides shows significant correlations between the usage of some words and replication (several such oligonucleotides are very significantly overrepresented in one of the strands and underrepresented in the other one).

Setting a statistical cut-off for the significance of duplications at 10^{-3} , we expected duplication by chance of words longer than 24 nucleotides to be rare²⁰. In fact, the genome of *B. subtilis* contains a plethora of such duplications, some of them appearing more than

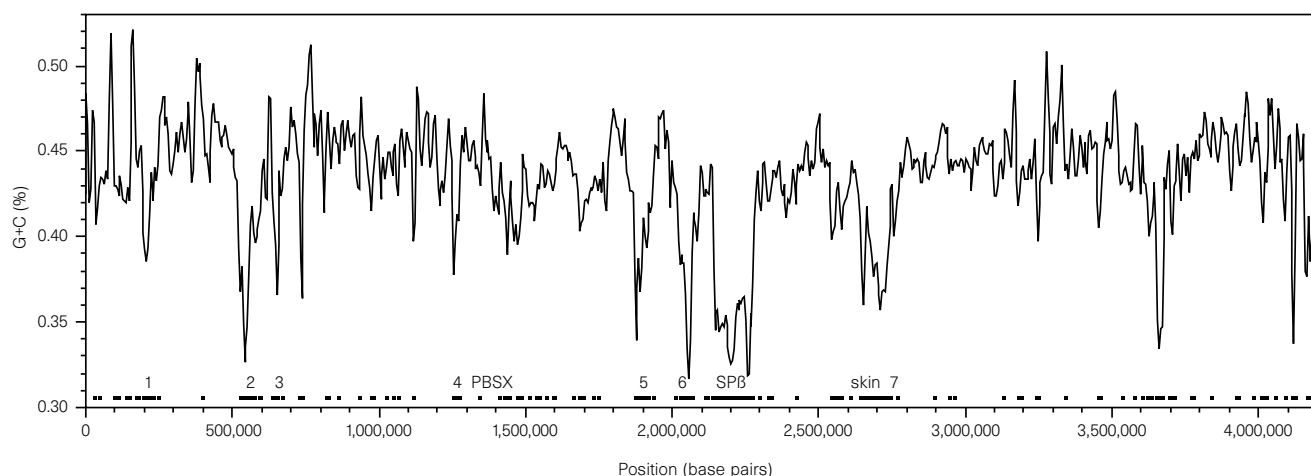


Figure 1 Distribution of A + T-rich islands along the chromosome of *B. subtilis*, in sliding windows of 10,000 nucleotides, with a step of 5,000 nucleotides. Location of genes from class 3 according to codon usage analysis (see Fig. 4) is indicated

by dots at the bottom of the graph. Known prophages (PBSX, SPB and *skin*) are indicated by their names, and prophage-like elements are numbered from 1 to 7.

twice. Among the duplications, we identified, as expected, the ribosomal RNA genes and their flanking regions, but also regions known to correspond to genes comprising long sequence repeats (such as *pks* and *srf*). We also found several regions that were not expected: a 182-bp repetition within the *yxaL* and *yxaO* genes; a 410-bp repetition between the *yxaK* and *yxaL* genes; an internal duplication of 174 bp inside *ycdI*; and significant duplications in the regions involved in the transcriptional control of several genes (such as 118 bp repeated three times between *yxbB* and *yxbC*). Finally, we found several repetitions at the borders of regions that might be involved in bacteriophage integration.

The most prominent duplication was a 190-bp element that was repeated 10 times in the chromosome. Multiple alignment of the ten repeats showed that they could be classified into two subfamilies with six and three copies each, plus a copy of what appears to be a chimera. Similar sequences have also been described in the closely related species *Bacillus licheniformis*^{21,22}. A striking feature of these repeats is that they are only found in half of the chromosome, at either side of the origin of replication, with five repeats on each side. Furthermore, with the exception of the most distal repeat at position 737,062, they lie in the same orientation with respect to the movement of the replication fork (Figs 2 and 3). Putative secondary structures conserved by compensatory mutations, as well as an insert in three of the copies, suggest that this element could indicate a structural RNA molecule.

Analysis at the transcription and translation level. Over 4,000 putative protein coding sequences (CDSs) have been identified, with an average size of 890 bp, covering 87% of the genome sequence (Fig. 2). We found that 78% of the genes started with ATG, 13% with TTG and 9% with GTG, which compares with 85%, 3% and 14%, respectively, in *E. coli*⁸. Fifteen genes (eight in the predicted CDSs in bacteriophage SP β) exhibiting unusual start codons (namely ATT and CTG) were also identified through their

similarities to known genes in other organisms or because they had a good GeneMark prediction (see Methods). This has not yet been substantiated experimentally. However, in the case of the gene coding for translation initiation factor 3, the similarity with its *E. coli* counterpart strongly suggests that the initiation codon is ATT, as is the case in *E. coli*.

We have not annotated CDSs that largely or entirely overlap existing genes, although such genes (for example, *comS* inside *srfAA*) certainly exist. It is also likely that some of the short CDSs present in the *B. subtilis* genome have been overlooked. For these reasons and possible sequencing errors, the estimated number of *B. subtilis* CDSs will fluctuate around the present figure of 4,100.

In several cases, in-frame termination codons or frameshifts were confirmed to be present on the chromosome (for example, an internal termination codon in *ywtF*, or the known programmed translational frameshift in *prfB*), indicating that the genes are either non-functional (pseudogenes) or subject to regulatory processes. It will therefore be of interest to determine whether these gene features are conserved in related *Bacillus* species, especially as strain 168 is derived from the Marburg strain that was subjected to X-ray irradiation²³.

A few regions do not have any identifiable feature indicating that they are transcribed: they could be 'grey holes' of the type described in *E. coli*²⁴. Preliminary studies involving all regions of more than 400 bp without annotated CDSs indicated that, of ~300 such regions, only 15% were likely to be really devoid of protein-coding sequences. One of the longest such regions, located between *yjfo* and *yjfn*, is 1,628 bp long. Grey holes seem generally to be clustered near the terminus of replication. However, a grey-hole cluster located at ~600 kb might be related to the temporary chromosome partition observed during the first stages of sporulation, when a segment of about one-third of the chromosome enters the prespore, and remains the sole part of the chromosome in the prespore for a significant transition period²⁵.

The codon usage of *B. subtilis* CDSs was analysed using factorial correspondence analysis¹⁷. We found that the CDSs of *B. subtilis* could be separated into three well-defined classes (Fig. 4). Class 1 comprises the majority of the *B. subtilis* genes (3,375 CDSs), including most of the genes involved in sporulation. Class 2 (188 CDSs) includes genes that are highly expressed under exponential growth conditions, such as genes encoding the transcription and translation machineries, core intermediary metabolism, stress proteins, and one-third of genes of unknown function. Class 3 (537 CDSs) contains a very high proportion of genes of unidentified function (84%), and the members of this class have codons enriched in A + T residues. These genes are usually clustered into groups between 15 and 160 genes (for example, bacteriophage SP β) and correspond to the A + T-rich islands described above (Fig. 1). When they are of known function, or when their products display similarity to proteins of known function, they usually correspond to functions found in, or associated with, bacteriophages or transposons, as well as functions related to the cell envelope. This includes the region *ycd/ycd/yde* (40 genes that are missing in some *B. subtilis* strains²⁶), where gene products showing similarities to bacteriophage and transposon proteins are intertwined. Many of these genes are associated with virulence genes identified in pathogenic Gram-positive bacteria, suggesting that such virulence factors are transmitted horizontally among bacteria at a much higher frequency than previously thought. If we include these A + T-rich regions as possible cryptic phages, together with known bacteriophages or bacteriophage-like elements (SP β , PBSX and the *skin* element), we find that the genome of *B. subtilis* 168 contains at least 10 such elements (Figs 2 and 3). Annotation of the corresponding regions often reveals the presence of genes that are similar to bacteriophage lytic enzymes, perhaps accounting for the observation that *B. subtilis* cultures are extremely prone to lysis.

The ribosomal RNA genes have been previously identified and

Table 1 Functional classification of the *Bacillus subtilis* protein-coding genes

The genes of known function or encoding products similar to known proteins in *B. subtilis* or in other organisms have been classified into functional categories (2,379 genes). The total number of genes in each category is indicated after the category title. Genes are listed in alphabetical order within each category, and their positions (in kilobases) on the *B. subtilis* chromosome are indicated after the gene names. A brief description is given for each gene. In some cases, interacting proteins have been indicated between brackets (for example, histidine kinases and response regulator, phosphatases and their substrates). More detailed and constantly updated information is available in the SubtiList database (see Methods). A preliminary assessment of the significance of sequence similarities was obtained through an automated procedure involving a combination between the BLAST2P probability and the percentage of amino-acid identity. Matches considered significant were re-examined manually. It should be emphasized that functions assigned to 'y' genes are based only on sequence similarity information with the best counterparts in protein databanks. Genes whose products are only similar to other unknown proteins, or not significantly similar to any other proteins in databanks (categories V and VI), were omitted.

Figure 2 General view of the *B. subtilis* chromosome. Arrows indicate the orientation of transcription. Genes are coloured according to their classification into six broad functional categories (blue, category I; green, category II; red, category III; orange, category IV; purple, category V; pink, category VI; see Table 1). Class 2 CDSs according to codon usage analysis are indicated by oblique hatches, and class 3 CDSs are indicated by vertical hatches. Ribosomal RNA genes are coloured in yellow. Transfer RNA genes are marked by triangles. Other RNA genes are represented as white arrows. Known genes (non-'y' genes) are printed in bold type. Putative transcription termination sites are represented as loops. Known prophages and prophage-like elements are indicated by brown hatches on the chromosome line. The 190-bp element repeated ten times is represented by hatched boxes.

shown to be organized into ten rRNA operons, mainly clustered around the origin of replication of the chromosome (Figs 2 and 3). In addition to the 84 previously identified tRNA genes, by using the Palingol²⁷ and tRNAscan²⁸ programs, we propose four putative new tRNA loci (at 1,262 kb, 1,945 kb, 2,003 kb and 2,899 kb), specific for lysine, proline and arginine (UUU, GGG, CCU and UCU anticodons, respectively). The 10S RNA involved in degradation of proteins made from truncated mRNA has been identified (*ssrA*), as well as the RNA component of RNase P (*rnpB*) and the 4.5S RNA involved in the secretion apparatus (*scr*).

There is a strong transcription orientation bias with respect to the movement of the replication fork: 75% of the predicted genes are transcribed in the direction of replication. Plotting the density of coding nucleotides in each strand along the chromosome readily identifies the replication origin and terminus (Fig. 3). To identify putative operons, we followed ref. 29 for describing Rho-independent transcription termination sites. This yielded ~1,630 putative terminators (340 of which were bidirectional). We retained only those that were located less than 100 bp downstream of a gene, or that were considered by the program to be 'very strong' (in order to account for possible erroneous CDSs). This yielded a total of ~1,250 terminators, with a mean operon size of three genes. A similar approach to the identification of promoters is problematical, especially because at least 14 sigma factors, recognizing different promoter sequences, have been identified in *B. subtilis*. Nevertheless, the consensus of the main vegetative sigma factor (σ^A) appears to be identical to its counterpart in *E. coli* (σ^{70}): 5'-TTGACA-*n*₁₇-TATAAT-3'. Relaxing the constraints of the similarity to sigma-specific consensus sequences led to an extremely high number of false-positive results, suggesting that the consensus-oriented approach to the identification of promoters should be replaced by another approach¹⁷.

Classification of gene products

Genes were classified according to ref. 14, based on the representation of cells as Turing machines in which one distinguishes between the machine and the program (Table 1). Using the BLAST2P software running against a composite protein databank compound of SWISS-PROT (release 34), TREMBL (release 3, update 1) and *B.*

subtilis proteins, we assigned at least one significant counterpart with a known function to 58% of the *B. subtilis* proteins. Thus for up to 42% of the gene products, the function cannot be predicted by similarity to proteins of known function: 4% of the proteins are similar only to other unknown proteins of *B. subtilis*; 12% are similar to unknown proteins from some other organism; and 26% of the proteins are not significantly similar to any other proteins in databanks. This preliminary analysis should be interpreted with caution, because only ~1,200 gene functions (30%) have been experimentally identified in *B. subtilis*. We used the 'y' prefix in gene names to emphasize that the function has not been ascertained (2,853 'y' genes, representing 70%).

Regulatory systems. Transcription regulatory proteins. Helix–turn–helix proteins form a large family of regulatory proteins found in both prokaryotes and eukaryotes. There are several classes, including repressors, activators and sigma factors. Using BLAST searches, we constructed consensus matrices for helix–turn–helix proteins to analyse the *B. subtilis* protein library. We identified 18 sigma or sigma-like factors, of which nine (including a new one) are of the SigA type. We also putatively identified 20 regulators (among which 18 were products of 'y' genes) of the GntR family, 19 regulators (15 'y' genes) of the LysR family, and 12 regulators (5 'y' genes) of the LacI family. Other transcription regulatory proteins were of the AraC family (11 members, 10 'y'), the Lrp family (7 members, 3 'y'), the DeoR family (6 members, 3 'y'), or additional families (such as the MarR, ArsR or TetR families). A puzzling observation is that several regulatory proteins display significant similarity to aminotransferases (seven such enzymes have been identified as showing similarity to repressors).

Two-component signal-transduction pathways. Two-component regulatory systems, consisting of a sensor protein kinase and a response regulator, are widespread among prokaryotes. We have identified 34 genes encoding response regulators in *B. subtilis*, most of which have adjacent genes encoding histidine kinases. Response regulators possess a well-conserved N-terminal phospho-acceptor domain³⁰, whereas their C-terminal DNA-binding domains share similarities with previously identified response regulators in *E. coli*, *Rhizobium meliloti*, *Klebsiella pneumoniae* or *Staphylococcus aureus*. Representatives of the four subfamilies recently identified in *E. coli*³¹

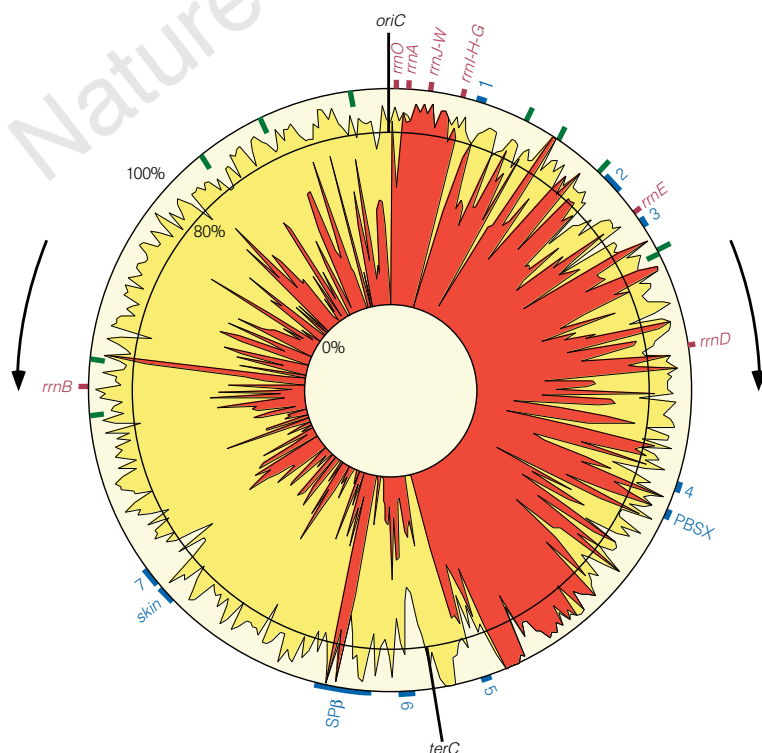


Figure 3 Density of coding nucleotides along the *B. subtilis* chromosome. Yellow stands for the density of coding nucleotides in both strands of the sequence; red indicates the density of coding nucleotides in the clockwise strand (nucleotides involved in genes transcribed in the clockwise orientation). The movement of the replication forks is represented by arrows. Ribosomal RNA operons are indicated by brown boxes. Known prophages and prophage-like elements are represented as blue lines. The 190-bp element repeated ten times is represented by green lines.

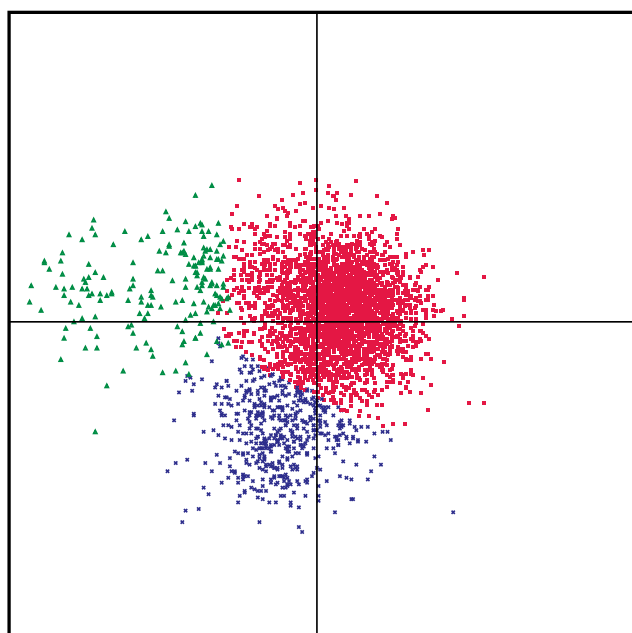


Figure 4 Factorial correspondence analysis of codon usage in the *B. subtilis* CDSs. Red dots, genes from class 1; green triangles, genes from class 2; blue crosses, genes from class 3. Class 2 contains genes coding for the translation and transcription machineries, and genes of the core intermediary metabolism. Class 3 genes correspond to codons strongly enriched in A or T in the wobble position; they generally belong to prophage-like inserts in the genome.

(OmpR, FixJ, CitB and LytR) have been identified in *B. subtilis*. In a fifth subfamily, CheY, the DNA-binding domain is absent. The DNA-binding domain of a single *B. subtilis* response regulator, YesN, shares similarity with regulatory proteins of the AraC family.

Quorum sensing. The *B. subtilis* genome contains 11 aspartate phosphatase genes, whose products are involved in dephosphorylation of response regulators, that do not seem to have counterparts in Gram-negative bacteria such as *E. coli*. Downstream from the corresponding genes are some small genes, called *phr*, encoding regulatory peptides that may serve as quorum sensors³². Seven *phr* genes have been identified so far, including three new genes (*phrG*, *phrI* and *phrK*).

Protein secretion. It is known that *B. subtilis* and related *Bacillus* species, in particular *B. licheniformis* and *B. amyloliquefaciens*, have a high capacity to secrete proteins into the culture medium. Several genes encoding proteins of the major secretion pathway have been identified: *secA*, *secD*, *secE*, *secF*, *secY*, *ffh* and *ftsY*. Surprisingly, there is no gene for the SecB chaperone. It is thought that other chaperone(s) and targeting factor(s), such as Ffh and FtsY, may take over the SecB function. Further, although there is only one such gene in *E. coli*, five type I signal peptidase genes (*sipS*, *sipT*, *sipU*, *sipV* and *sipW*) have been found³³. The *lsp* gene, encoding a type II signal peptidase required for processing of lipo-modified precursors, was also identified. PrsA, located at the outer side of the membrane, is important for the refolding of several mature proteins after their translocation through the membrane.

Other families of proteins. ABC transporters were the most frequent class of proteins found in *B. subtilis*. They must be extremely important in Gram-positive bacteria, because they have an envelope comprising a single membrane. ABC transporters will therefore allow such bacteria to escape the toxic action of many compounds. We propose that 77 such transporters are encoded in the genome. In general they involve the interaction of at least three gene products, specified by genes organized into an operon. Other families comprised 47 transport proteins similar to facilitators (and perhaps sometimes part of the ABC transport systems), 18 amino-acid permeases (probably antiporters), and at least 16 sugar transporters belonging to the PEP-dependent phosphotransferase system.

General stress proteins are important for the survival of bacteria under a variety of environmental conditions. We identified 43 temperature-shock and general stress proteins displaying strong similarity to *E. coli* counterparts.

Missing genes. Histone-like proteins such as HU and H-NS have been identified in *E. coli*. We found that *B. subtilis* encodes two putative histone-like proteins that show similarity to *E. coli* HU, namely HBSu and YonN, but found no homologue to H-NS. It is known that the *hbs* gene encoding HBSu is essential, but we do not expect the *yonN* gene to be essential because it is present in the SP β prophage. IHF is similar to HU, and it is not known whether HBSu plays a similar role to that of IHF in *E. coli*. Similarly, no protein similar to FIS could be found.

Genes encoding products that interact with methylated DNA, such as *seqA* in *E. coli*, involved in the regulation of replication initiation timing, or *mutH*, the endonuclease recognizing the newly synthesized strand during mismatch repair at hemi-methylated

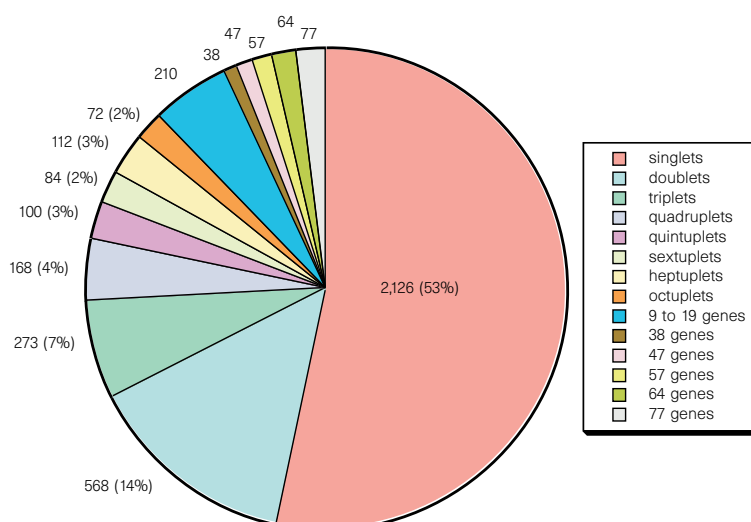


Figure 5 Gene paralogue distribution in the genome of *B. subtilis*. Each *B. subtilis* protein has been compared with all other proteins in the genome, using a Smith and Waterman algorithm. The baseline is established by making a similar

comparison using 100 independent random shuffles of the protein sequence (Z-score > 13).

GATC sites, are also missing. This is in line with the absence of known methylation in *B. subtilis*, equivalent to Dam methylation in *E. coli*. Similarly, *E. coli* *sfiA*, encoding an inhibitor of FtsZ action in the SOS response, has no counterpart in *B. subtilis*. In contrast, *B. subtilis* replication initiation-specific genes, such as *dnaB* and *dnaD*, are missing in *E. coli*. The exact counterpart of the *E. coli* *mukB* gene, involved in chromosome partitioning, does not exist in *B. subtilis*, but genes *spo0J* and *smc* (Smc is weakly similar to MukB), which are suggested to be involved in partitioning of the *B. subtilis* chromosome, are missing in *E. coli*.

Turnover of mRNA is controlled in *E. coli* by a 'degradosome' comprising RNase E. It has a counterpart in *B. subtilis*, but we failed to find a clear homologue of RNase E in this organism. Whether this is related to the role of ribosomal protein S1 as an RNA helicase involved in mRNA turnover in *E. coli* requires further investigation. In particular, a homologue of *rpsA* (S1 structural gene), *ypfD*, might be involved in a structure homologous to the degradosome³⁴.

Structurally unrelated genes of similar function. Several genes encode products that have similar functions in *E. coli* and *B. subtilis*, but have no evident common structure. This is the case for the helicase loader genes, *E. coli* *dnaC* and *B. subtilis* *dnaI*; the genes coding for the replication termination protein, *E. coli* *tus* and *B. subtilis* *rtp*; and the division topology specifier genes, *E. coli* *minE* and *B. subtilis* *divIVA*. The situation may even be more complex in multisubunit enzymes: *B. subtilis* synthesizes two DNA polymerase III α chains, one having 3'–5' proofreading exonuclease activity (PolC) and the other without the exonuclease activity (DnaE); in *E. coli*, only the latter exists. *E. coli* DNA polymerase II is structural, related to DNA polymerase α of eukaryotes, whereas *B. subtilis* YshC is related to DNA polymerase β .

Metabolism of small molecules

The type and range of metabolism used for the interconversion of low-molecular-weight compounds provide important clues to an organism's natural environment(s) and its biological activity. Here we briefly outline the main metabolic pathways of *B. subtilis* before the reconstruction of these pathways *in silico*, the correlation of genes with specific steps in the pathway, and ultimately the prediction of patterns of gene expression.

Intermediary metabolism. It has long been known that *B. subtilis* can use a variety of carbohydrates. As expected, it encodes an Embden–Meyerhof–Parnas glycolytic pathway, coupled to a functional tricarboxylic acid cycle. Further, *B. subtilis* is also able to grow anaerobically in the presence of nitrate as an electron acceptor. This metabolism is, at least in part, regulated by the FNR protein, binding to sites upstream of at least eight genes (four sites experimentally confirmed and four putative sites). A noteworthy feature of *B. subtilis* metabolism is an apparent requirement of branched short-chain carboxylic acids for lipid biosynthesis³⁵. Branched-chain 2-keto acid decarboxylase activity exists and may be linked to a variety of genes, suggesting that *B. subtilis* can synthesize and utilize linear branched short-chain carboxylic acids and alcohols.

Amino-acid and nucleotide metabolism. Pyrimidine metabolism of *B. subtilis* seems to be regulated in a way fundamentally different from that of *E. coli*, as it has two carbamylphosphate synthetases (one specific for arginine synthesis, the other for pyrimidine). Additionally, the aspartate transcarbamylase of *B. subtilis* does not act as an allosteric regulator as it does in *E. coli*. As in other microorganisms, pyrimidine deoxyribonucleotides are synthesized from ribonucleoside diphosphates, not triphosphates. The cytidine diphosphate required for DNA synthesis is derived from either the salvage pathway of mRNA turnover or from the synthesis of phospholipids and components of the cell wall. This means that polynucleotide phosphorylase is of fundamental importance in nucleic acid metabolism, and may account for its important role in competence³⁶. Two ribonucleoside reductases, both of class I, NrdEF type, are encoded by the *B. subtilis* chromosome, in one case

from within the SP β genome. In this latter case, the gene corresponding to the large subunit both contains an intron and codes for an intein (V.L., unpublished data). The gene of the small subunit of this enzyme also contains an intron, encoding an endonuclease, as was found for the homologue in bacteriophage T4.

By similarity with genes from other organisms, there appears to be, in addition to genes involved in amino-acid degradation (such as the *roc* operon, which degrades arginine and related amino acids), a large number of genes involved in the degradation of molecules such as opines and related molecules, derived from plants. This is also in line with the fact that *B. subtilis* degrades polygalacturonate, and suggests that, in its biotope, it forms specific relations with plants.

Secondary metabolism. In addition to many genes coding for degradative enzymes, almost 4% of the *B. subtilis* genome codes for large multifunctional enzymes (for example, the *srf*, *pps* and *pks* loci), similar to those involved in the synthesis of antibiotics in other genera of Gram-positive bacteria such as *Streptomyces*. Natural isolates of *B. subtilis* produce compounds with antibiotic activity, such as surfactin, fengycin and difficidin, that can be related to the above-mentioned loci. This bacterium therefore provides a simple and genetically amenable model in which to study the synthesis of antibiotics and its regulation. These pathways are often organized in very long operons (for example, the *pks* region spans 78.5 kb, about 2% of the genome). The corresponding sequences are mostly located near the terminus of replication, together with prophages and prophage-like sequences.

Paralogues and orthologues

It is important to relate intermediary metabolism to genome structure, function and evolution. We therefore compared the *B. subtilis* proteins with themselves, as well as with proteins from known complete genomes, using a consistent statistical method that allows the evaluation of unbiased probabilities of similarities between proteins^{37,38}. For *Z*-scores higher than 13, the number of proteins similar to each given protein does not vary, indicating that this cut-off value identifies sets of proteins that are significantly similar.

Families of paralogues. Many of the paralogues constitute large families of functionally related proteins, involved in the transport of compounds into and out of the cell, or involved in transcription regulation. Another part of the genome consists of gene doublets (568 genes), triplets (273 genes), quadruplets (168 genes) and quintuplets (100 genes). Finally, about half of the genome is made of genes coding for proteins with no apparent paralogues (Fig. 5). No large family comprises only proteins without any similarity to proteins of known function.

The process by which paralogues are generated is not well understood, but we might find clues by studying some of the duplications in the genome. Several approximate DNA repetitions, associated with very high levels of protein identity, were found, mainly within regions putatively or previously identified as prophages. This is in line with previous observations about PBSX and the *skin* element^{39,40}, and suggests that these prophage-like elements share a common ancestor and have diverged relatively recently. In addition, several protein duplications are in genes that are located very close to each other, such as *yukL* and *dhbF* (the corresponding proteins are 65% identical in an overlap of 580 amino acids), *yugJ* and *yugK* (proteins 73% identical), *yxjG* and *yxjH* (proteins 70% identical), and the entire *opuB* operon, which is duplicated 3 kb away (*opuC* operon, yielding ~80% of amino-acid identity in the corresponding proteins).

The study of paralogues showed that, as in other genomes, a few classes of genes have been highly expanded. This argues against the idea of the genome evolving through a series of duplications of ancestral genomes, but rather for the idea of genes as living organisms, subject to evolutionary constraints, some being sub-

mitted to expansion and natural selection, and others to local duplications of DNA regions.

Among paralogue doublets, some were unexpected, such as the three aminoacyl tRNA synthetases doublets (*hisS* (2,817 kb) and *hisZ* (3,588 kb); *thrS* (2,960 kb) and *thrZ* (3,855 kb); *tyrS* (3,036 kb) and *tyrZ* (3,945 kb)) or the two *mutS* paralogues (*mutS* and *yshD*). This latter situation is similar to that found in *Synechocystis*. In the case of *B. subtilis*, the presence of two MutS proteins could indicate that there are two different pathways for long-patch mismatch repair, possibly a consequence of the active genetic transformation mechanism of *B. subtilis*.

Families of orthologues. Because *Mycoplasma* spp. are thought to be derived from Gram-positive bacteria similar to *B. subtilis*, we compared the *B. subtilis* genome with that of *M. genitalium*. Among the 450 genes encoded by *M. genitalium*, the products of 300 are similar to proteins of *B. subtilis*. Among the 146 remaining gene products, a further 3 are similar to proteins of other *Bacillus* species, and 9 to proteins of other Gram-positive bacteria; 25 are similar to proteins of Gram-negative bacteria; and 19 are similar to proteins of other *Mycoplasma* spp. This leaves only 90 genes that would be specific to *M. genitalium* and might be involved in the interaction of this organism with its host.

The *B. subtilis* genome is similar in size to that of *E. coli*. Because these bacteria probably diverged more than one billion years ago, it is of evolutionary value to investigate their relative similarity. About 1,000 *B. subtilis* genes have clear orthologous counterparts in *E. coli* (one-quarter of the genome). These genes did not belong either to the prophage-like regions or to regions coding for secondary metabolism (~15% of the *B. subtilis* genome). This indicates that a large fraction of these genomes shared similar functions. At first sight, however, it seems that little of the operon structure has been conserved. We nevertheless found that ~100 putative operons or parts of operons were conserved between *E. coli* and *B. subtilis*. Among these, ~12 exhibited a reshuffled gene order (typically, the arabinose operon is *araBAD* in *B. subtilis* and *araBAD* in *E. coli*). In addition to the core of the translation and transcription machinery, we identified other classes of operons that were well conserved between the two organisms, including major integrated functions such as ATP synthesis (*atp* operon) and electron transfer (*cta* and *qox* operons). As well as being well preserved, the murein biosynthetic region was partly duplicated, allowing creation of part of the genes required for the sporulation division machinery⁴¹. The amino-acid biosynthesis genes differ more in their organization: the *E. coli* genes for arginine biosynthesis are spread throughout the chromosome, whereas the arginine biosynthesis genes of *B. subtilis* form an operon. The same is true for purine biosynthetic genes. Genes responsible for the biosynthesis of coenzymes and prosthetic groups in *B. subtilis* are often clustered in operons that differ from those found in *E. coli*. Finally, several operons conserved in *E. coli* and *B. subtilis* correspond to unknown functions, and should therefore be priority targets for the functional analysis of these model genomes.

Comparison with *Synechocystis* PCC6803 revealed about 800 orthologues. However, in this case the putative operon structure is extremely poorly conserved, apart from four of the ribosomal protein operons, the *groES*–*groEL* operon, *yfnHG* (respectively in *Synechocystis* *rfbFG*), *rpsB-ts*, *ylxS-nusA-infB*, *asd-dapGA-ymfA*, *spmAB*, *efp-accB*, *grpE-dnaK*, *yurXW*. The nine-gene *atp* operon of *B. subtilis* is split into two parts in *Synechocystis*: *atpBE* and *atpIHGFDAC*.

Conclusion

The biochemistry, physiology and molecular biology of *B. subtilis* have been extensively studied over the past 40 years. In particular, *B. subtilis* has been used to study postexponential phase phenomena such as sporulation and competence for DNA uptake. The genome sequences of *E. coli* and *B. subtilis* provide a means of studying the

evolutionary divergence, one billion years ago, of eubacteria into the Gram-positive and Gram-negative groups. The availability of powerful genetic tools will allow the *B. subtilis* genome sequence data to be exploited fully within the framework of a systematic functional analysis program, undertaken by a consortium of 19 European and 7 Japanese laboratories coordinated by S. D. Ehrlich (INRA, Jouy-en-Josas, France) and by N. Ogasawara and H. Yoshikawa (Nara Institute of Science and Technology, Nara, Japan). □

Methods

Genome cloning and sequencing. An international consortium was established to sequence the genome of *B. subtilis* strain 168 (refs 9, 10, 42). At its peak, 25 European, seven Japanese and one Korean laboratory participated in the program, together with two biotechnology companies. Five contiguous DNA regions totalling 0.94 Mb, and two additional regions of 0.28 and 0.14 Mb, were sequenced by the Japanese partners, while the European partners sequenced a total of 2.68 Mb. A few sequences from strain 168 published previously were not resequenced when long overlaps did not indicate differences.

A major technical difficulty was the inability to construct in *E. coli* gene banks representative of the entire *B. subtilis* chromosome using vectors that have proved efficient for other sources of bacterial DNA (such as bacteriophage or cosmid vectors). This was due to the generally very high level of expression of *B. subtilis* genes in *E. coli*, leading to toxic effects. This limitation was overcome by: cloning into a variety of vectors^{9,43,44}; using an *E. coli* strain maintaining low-copy number plasmids⁴⁴; using an integrative plasmid/marker rescue genome-walking strategy⁴⁴; and *in vitro* amplification using polymerase chain reaction (PCR) techniques^{45,46}.

Although cloning vectors were used in the early stages as templates for sequencing reactions, they were largely superseded in the later stages by long-range and inverse PCR techniques. To reduce sequencing errors resulting from PCR amplification artefacts, at least eight amplification reactions were performed independently and subsequently pooled. The various sequencing groups were free to choose their own strategy, except that all DNA sequences had to be determined entirely on both strands.

Sequence annotation and verification. The sequences were annotated by the groups, and sent to a central depository at the Institut Pasteur¹⁴. The Japanese sequences were also sent there through the Japanese depository at the Nara Institute of Science and Technology. The same procedures were used to identify CDSs and to detect frameshifts. They were embedded within a cooperative computer environment dedicated to automatic sequence annotation and analysis³⁹. In a first step, we identified in all six possible frames the open reading frames (ORFs) that were at least 100 codons in length. In a second step, three independent methods were used: the first method used the GeneMark coding-sequence prediction method⁴⁷ together with the search for CDSs preceded by typical translation initiation signals (5'-AAGGAGGTG-3'), located 4–13 bases upstream of the putative start codons (ATG, TTG or GTG); the second method used the results of a BLAST2X analysis performed on the entire *B. subtilis* genome against the non-redundant protein database at the NCBI; and the third method was based on the distribution of non-overlapping trinucleotides or hexanucleotides in the three frames of an ORF⁴⁸.

In general, frameshifts and missense mutations generating termination codons or eliminating start codons are relatively easy to detect. We shall devise a procedure for detecting another type of error, GC instead of CG or vice versa, which are much more difficult to identify. It should be noted that putative frameshift errors should not be corrected automatically. The sequences of the flanking regions of a 500-bp fragment centred around a putative error were sent to an independent verification group, which performed PCR amplifications using chromosomal DNA as template, and sequenced the corresponding DNA products.

Organization and accessibility of data. The *B. subtilis* sequence data have been combined with data from other sources (biochemical, physiological and genetic) in a specialized database, SubtiList⁴⁹, available as a Macintosh or Windows stand-alone application (4th Dimension runtime) by anonymous FTP at <ftp://ftp.pasteur.fr/pub/GenomeDB/SubtiList>. SubtiList is also accessible through a World-Wide Web server at <http://www.pasteur.fr/Bio/SubtiList.html>.

where it has been implemented on a UNIX system using the Sybase relational database management system. A completely rewritten version of SubtiList is in preparation to facilitate browsing of the information of the whole chromosome. Flat files of the whole DNA and protein sequences in EMBL and FASTA format will be made available at the above ftp address. Another *B. subtilis* genome database is also under development at the Human Genome Center of Tokyo University (<http://www.genome.ad.jp>), and SubtiList will also be available there.

Received 16 July; 29 September 1997.

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Acknowledgements. We thank C. Anagnostopoulos, R. Dedonder and J. Hoch for their pioneering efforts, and A. Bairoch for advice in annotating *B. subtilis* protein data. The main funding of the European network was provided by the European Commission under the Biotechnology program. The Japanese project was included in the Human Genome Program, and supported by a research grant from the Ministry of Education, Science and Culture, and the Proposal-Based Advanced Industrial Technology R&D Program from New Energy and Industrial Technology Development Organization. The Swiss and Korean projects were funded by the Swiss National Fund and the Korean government, respectively. An industrial platform was set up to facilitate contacts between participants of the European consortium and some European biotechnology companies: DuPont de Nemours (France, USA), Frimond (Belgium), Genecor (Finland, USA), Gist Brocades (The Netherlands), Glaxo-Wellcome (UK, Italy), Hoechst Marion Roussel (France, Germany), F. Hoffmann-La Roche AG (Switzerland), Novo Nordisk (Denmark), SmithKline Beecham (UK).

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<i>ydeR</i>	578	antibiotic resistance protein	<i>ytmJ</i>	3007	amino acid ABC transporter (binding protein)	<i>yciK</i>	427	two-component sensor histidine kinase [YciJ]
<i>ydeA</i>	580	arsenical pump membrane protein	<i>ytmK</i>	3006	amino acid ABC transporter (binding protein)	<i>ydbF</i>	497	two-component sensor histidine kinase [YdbG]
<i>ydfJ</i>	589	antibiotic transport-associated protein	<i>ytmL</i>	3005	amino acid ABC transporter (permease)	<i>ydfH</i>	587	two-component sensor histidine kinase [YdfI]
<i>ydlH</i>	595	multidrug-efflux transporter regulator	<i>ytmM</i>	3005	amino acid ABC transporter (permease)	<i>yesM</i>	758	two-component sensor histidine kinase [YesN]
<i>ydlM</i>	596	cation efflux system	<i>ytnA</i>	3125	proline permease	<i>yfiJ</i>	903	two-component sensor histidine kinase [YfiK]
<i>ydlO</i>	597	ABC transporter (binding protein)	<i>ytrE</i>	3118	ABC transporter (ATP-binding protein)	<i>yhcY</i>	1008	two-component sensor histidine kinase [YhcZ]
<i>ydgF</i>	608	amino acid ABC transporter (permease)	<i>ytrB</i>	3115	ABC transporter (ATP-binding protein)	<i>yhlJ</i>	1129	sensory transduction pleiotropic regulatory protein
<i>ydgH</i>	609	transporter	<i>ytsC</i>	3111	ABC transporter (ATP-binding protein)			
<i>ydgK</i>	613	bicyclomycin resistance protein	<i>ytsD</i>	3110	ABC transporter (permease)	<i>ykoH</i>	1392	two-component sensor histidine kinase [YkoG]
<i>ydhL</i>	626	chloramphenicol resistance protein	<i>ytsB</i>	3108	multidrug resistance protein	<i>ykrQ</i>	1419	two-component sensor histidine kinase
<i>ydhM</i>	626	cellobiose phosphotransferase system enzyme II	<i>yubD</i>	3192	multidrug resistance protein	<i>ykvD</i>	1432	two-component sensor histidine kinase
<i>ydhN</i>	627	cellobiose phosphotransferase system enzyme II	<i>yubG</i>	3188	Na ⁺ -transporting ATP synthase	<i>yocF</i>	2090	two-component sensor histidine kinase [YocG]
<i>ydhO</i>	627	cellobiose phosphotransferase system enzyme II	<i>yufN</i>	3239	ABC transporter (lipoprotein)	<i>ykrQ</i>	2704	two-component sensor histidine kinase [YkrP]
<i>ydlF</i>	648	ABC transporter (ATP-binding protein)	<i>yufO</i>	3240	ABC transporter (ATP-binding protein)	<i>ytrP</i>	3035	two-component sensor histidine kinase
<i>ydlD</i>	668	H ⁺ -symporter	<i>yufR</i>	3244	organic acid transport protein	<i>ytsB</i>	3112	two-component sensor histidine kinase
<i>ydlK</i>	676	sugar transporter	<i>yufU</i>	3248	Na ⁺ /H ⁺ antiporter	<i>yutL</i>	3236	two-component sensor histidine kinase [YutM]
<i>ydeA</i>	687	cation efflux system membrane protein	<i>yufV</i>	3249	Na ⁺ /H ⁺ antiporter	<i>yvcQ</i>	3566	two-component sensor histidine kinase [YvcP]
<i>yecA</i>	712	amino acid permease	<i>yugO</i>	3218	potassium channel protein	<i>yvtT</i>	3497	two-component sensor histidine kinase [YvtU]
<i>yesO</i>	761	sugar-binding protein	<i>yunJ</i>	3330	purine permease	<i>yvqB</i>	3385	two-component sensor histidine kinase [YvtU]
<i>yesP</i>	762	lactose permease	<i>yunK</i>	3331	purine permease	<i>yvqE</i>	3395	two-component sensor histidine kinase [YvqF]
<i>yesQ</i>	763	lactose permease	<i>yurJ</i>	3345	multiple sugar ABC transporter (ATP-binding protein)	<i>yvrG</i>	3407	two-component sensor histidine kinase [YvrH]
<i>yfhA</i>	921	iron(III) diclimate transport permease				<i>ywpD</i>	3741	two-component sensor histidine kinase
<i>yfhI</i>	926	antibiotic resistance protein	<i>yurM</i>	3348	sugar permease	<i>yxdK</i>	4071	two-component sensor histidine kinase [YxdJ]
<i>yfiB</i>	893	ABC transporter (ATP-binding protein)	<i>yurN</i>	3349	sugar permease	<i>yxiM</i>	3992	two-component sensor histidine kinase [YxiL]
<i>yfiC</i>	895	ABC transporter (ATP-binding protein)	<i>yurO</i>	3350	multiple sugar-binding protein	<i>yycG</i>	4153	two-component sensor histidine kinase [YycF]
<i>yfiG</i>	900	metabolite transport protein	<i>yurY</i>	3360	ABC transporter (ATP-binding protein)			
<i>yfiL</i>	905	ABC transporter (ATP-binding protein)	<i>yusC</i>	3363	ABC transporter (ATP-binding protein)			
<i>yfiM</i>	906	ABC transporter (ATP-binding protein)	<i>yusP</i>	3374	multidrug-efflux transporter			
<i>yfiN</i>	907	ABC transporter (ATP-binding protein)	<i>yusV</i>	3379	iron(III) diclimate transport permease			
<i>yfiS</i>	913	multidrug resistance protein	<i>yutK</i>	3307	Na ⁺ /nucleoside cotransporter			
<i>yfiU</i>	916	multidrug-efflux transporter	<i>yuxJ</i>	3232	multidrug-efflux transporter			
<i>yfiY</i>	920	iron(III) diclimate transport permease	<i>yvaE</i>	3448	multidrug-efflux transporter			
<i>yfiZ</i>	920	iron(III) diclimate transport permease	<i>yvbW</i>	3490	amino acid permease			
<i>yjiQ</i>	872	divalent cation transport protein	<i>yvcC</i>	3579	ABC transporter (ATP-binding protein)			
<i>yjKE</i>	865	H ⁺ /Ca ²⁺ exchanger	<i>yvcR</i>	3565	ABC transporter (ATP-binding protein)			
<i>yjKF</i>	865	multidrug-efflux transporter	<i>yvcS</i>	3565	ABC transporter (permease)			
<i>yjKH</i>	862	transporter	<i>yvdB</i>	3561	transporter			
<i>yjKL</i>	861	multidrug resistance protein	<i>yvdG</i>	3555	maltose/maltodextrin-binding protein			
<i>yjLA</i>	844	amino acid carrier protein	<i>yvdH</i>	3554	maltodextrin transport system permease			
<i>yjLE</i>	844	anion-binding protein	<i>yvdI</i>	3552	maltodextrin transport system permease			
<i>yjLF</i>	840	phosphotransferase system enzyme II	<i>yvE</i>	3538	permease			
<i>yjLS</i>	829	2-oxoglutarate/maleate translocator	<i>yvH</i>	3510	L-lactate permease			
<i>yfmC</i>	826	ferrichrome ABC transporter (binding protein)	<i>yvK</i>	3508	maltose/maltodextrin-binding protein			
<i>yfmD</i>	825	ferrichrome ABC transporter (permease)	<i>yvL</i>	3506	maltodextrin transport system permease			
<i>yfmE</i>	824	ferrichrome ABC transporter (permease)	<i>yvM</i>	3505	maltodextrin transport system permease			
<i>yfmF</i>	824	ferrichrome ABC transporter (ATP-binding protein)	<i>yvR</i>	3498	ABC transporter (ATP-binding protein)			
<i>yfmM</i>	815	ABC transporter (ATP-binding protein)	<i>yvX</i>	3424	molybdenum-binding protein			
<i>yfmO</i>	812	multidrug-efflux transporter	<i>yvL</i>	3424	molybdate-binding protein			
<i>yfmR</i>	809	ABC transporter (ATP-binding protein)	<i>yvM</i>	3425	molybdenum transport permease			
<i>yfnA</i>	806	metabolite transporter	<i>yvW</i>	3440	heavy metal-transporting ATPase			
<i>ygaD</i>	939	ABC transporter (ATP-binding protein)	<i>yvX</i>	3443	heavy metal-transporting ATPase			
<i>ygaL</i>	961	nitrate ABC transporter (binding protein)	<i>yvY</i>	3443	mercuric transport protein			
<i>ygaM</i>	963	ABC transporter (permease)	<i>yvA</i>	3618	multidrug-efflux transporter			
<i>ygbA</i>	962	ABC transporter (binding lipoprotein)	<i>yvmA</i>	3605	transporter			
<i>yhaQ</i>	1062	ABC transporter (ATP-binding protein)	<i>yvJ</i>	3399	macrolide-efflux protein			
<i>yhaU</i>	1060	Na ⁺ /H ⁺ antiporter	<i>yvR</i>	3402	iron transport system			
<i>yhcA</i>	977	multidrug resistance protein	<i>yvB</i>	3403	iron permease			
<i>yhgG</i>	981	glycine betaine/L-proline transport	<i>yvC</i>	3403	iron-binding protein			
<i>yhdH</i>	982	ABC transporter (ATP-binding protein)	<i>yvD</i>	3413	amino acid ABC transporter (ATP-binding protein)			
<i>yhcI</i>	984	ABC transporter (binding lipoprotein)	<i>yvH</i>	3420	ABC transporter (amino acid permease)			
<i>yhdL</i>	986	sodium-glutamate symporter	<i>ywbA</i>	3938	phosphotransferase system enzyme II			
<i>yhdG</i>	1023	amino acid transporter	<i>ywbF</i>	3933	sugar permease			
<i>yhdH</i>	1024	sodium-dependent transporter	<i>ywcA</i>	3923	Na ⁺ -dependent symport			
<i>yheH</i>	1047	ABC transporter (ATP-binding protein)	<i>yvcl</i>	3904	nitrite transporter			
<i>yheL</i>	1045	ABC transporter (ATP-binding protein)	<i>ywA</i>	3874	chloramphenicol resistance			
<i>yheL</i>	1044	Na ⁺ /H ⁺ antiporter	<i>ywF</i>	3869	efflux protein			
<i>yhiQ</i>	1107	iron(III) diclimate-binding protein	<i>ywhQ</i>	3837	ABC transporter (ATP-binding protein)			
<i>yhiB</i>	1102	metabolite permease	<i>ywA</i>	3821	ABC transporter (ATP-binding protein)			
<i>yhiO</i>	1133	multidrug-efflux transporter	<i>ywoA</i>	3758	bactericin transport permease			
<i>yhiP</i>	1133	transporter binding protein	<i>ywC</i>	3754	transporter			
<i>yitG</i>	1177	multidrug resistance protein	<i>ywoE</i>	3753	permease			
<i>yitZ</i>	1194	multidrug resistance protein	<i>ywoG</i>	3749	antibiotic resistance protein			
<i>yjBQ</i>	1240	Na ⁺ /H ⁺ antiporter	<i>ywpC</i>	3743	large conductance mechanosensitive channel protein			
<i>yjdD</i>	1272	fructose phosphotransferase system enzyme II						
<i>yjKB</i>	1296	amino acid ABC transporter (ATP-binding protein)	<i>ywrA</i>	3721	chromate transport protein			
<i>yjmB</i>	1301	Na ⁺ /galactoside symporter	<i>ywrB</i>	3720	chromate transport protein			
<i>yjmG</i>	1307	hexuronate transporter	<i>ywrK</i>	3712	arsenical pump membrane protein			
<i>ykaB</i>	1350	low-affinity inorganic phosphate transporter	<i>ywtG</i>	3693	metabolite transport protein			
<i>ykaB</i>	1352	amino acid permease	<i>yxmM</i>	4100	antibiotic resistance protein			
<i>ykaC</i>	1353	ABC transporter (binding protein)	<i>yxmC</i>	4087	metabolite transport protein			
<i>ykdD</i>	1368	propeptide ABC transporter (permease)	<i>yxL</i>	4070	ABC transporter (ATP-binding protein)			
<i>yknU</i>	1499	ABC transporter (ATP-binding protein)	<i>yxdM</i>	4069	ABC transporter (permease)			
<i>yknV</i>	1501	ABC transporter (ATP-binding protein)	<i>yxB</i>	4066	ABC transporter (binding protein)			
<i>yknY</i>	1505	ABC transporter (ATP-binding protein)	<i>yxeM</i>	4059	amino acid ABC transporter (binding protein)			
<i>ykoD</i>	1390	cation ABC transporter (ATP-binding protein)	<i>yxeN</i>	4058	amino acid ABC transporter (permease)			
<i>ykoK</i>	1395	Mg ²⁺ transporter	<i>yxeO</i>	4058	amino acid ABC transporter (ATP-binding protein)			
<i>ykpA</i>	1512	ABC transporter (ATP-binding protein)	<i>yxeR</i>	4054	ethanolamine transporter			
<i>ykrM</i>	1416	Na ⁺ -transporting ATP synthase	<i>yxiQ</i>	4009	Mg ²⁺ /citrate complex transporter			
<i>ykuC</i>	1476	macrolide-efflux protein	<i>yxjA</i>	4005	pyrimidine nucleoside transport			
<i>ykvW</i>	1451	heavy metal-transporting ATPase	<i>yxjK</i>	3979	metabolite-sodium symport			
<i>ylmA</i>	1608	ABC transporter (ATP-binding protein)	<i>yxjL</i>	3970	purine-cytosine permease			
<i>yloA</i>	1630	anion permease	<i>yxiF</i>	3968	ABC transporter (ATP-binding protein)			
<i>yloB</i>	1637	calcium-transporting ATPase	<i>yxiH</i>	3966	multidrug-efflux transporter			
<i>ynaL</i>	1887	H ⁺ -symporter	<i>yxiJ</i>	4194	transporter			
<i>yncC</i>	1896	metabolite transport protein	<i>yjBF</i>	4180	antibiotic resistance protein			
<i>yocN</i>	2098	permease	<i>yjbl</i>	4175	ABC transporter (ATP-binding protein)			
<i>yocR</i>	2106	sodium-dependent transporter	<i>yjBL</i>	4174	ABC transporter (permease)			
<i>yocS</i>	2106	sodium-dependent transporter	<i>yjBO</i>	4169	ABC transporter (permease)			
<i>yodE</i>	2129	aromatic metabolite transporter	<i>yjCB</i>	4159	ABC transporter (permease)			
<i>yodF</i>	2130	proline permease	<i>yjDL</i>	4125	ABC transporter (ATP-binding protein)			
<i>yojA</i>	2125	glucanate permease	<i>yjZE</i>	4122	phosphotransferase system enzyme II			
<i>ypeE</i>	2337	phosphotransferase system enzyme II						
<i>ypeV</i>	2620	Na ⁺ /P _i cotransporter						
<i>yqgG</i>	2581	phosphate ABC transporter (binding protein)						
<i>yqgH</i>	2580	phosphate ABC transporter (permease)						
<i>yqgl</i>	2579	phosphate ABC transporter (permease)						
<i>yqal</i>	2578	phosphate ABC transporter (ATP-binding protein)						
<i>yqgK</i>	2577	phosphate ABC transporter (ATP-binding protein)						
<i>yqIH</i>	2515	lipoprotein						
<i>yqiX</i>	2492	amino acid ABC transporter (binding protein)						
<i>yqiY</i>	2491	amino acid ABC transporter (permease)						
<i>yqIZ</i>	2491	amino acid ABC transporter (ATP-binding protein)						
<i>yqIV</i>	2466	multidrug resistance protein						
<i>yqKI</i>	2453	Na ⁺ /H ⁺ antiporter						
<i>yraC</i>	2745	citrate transporter						
<i>yrbD</i>	2841	sodium/proton-dependent alanine carrier protein						
<i>ytcP</i>	3087	ABC transporter (permease)						
<i>ytcQ</i>	3086	lipoprotein						
<i>yteQ</i>	3082	sugar transport protein						
<i>ytgA</i>	3145	ABC transporter (membrane protein)						
<i>ytgB</i>	3144	ABC transporter (ATP-binding protein)						
<i>ytgC</i>	3143	ABC transporter (membrane protein)						
<i>ythP</i>	3071	ABC transporter (ATP-binding protein)						
<i>ytiC</i>	3132	anion transport ABC transporter (ATP-binding protein)						
<i>ytiD</i>	3133	ABC transporter (permease)						
<i>ytiP</i>	3065	ABC transporter (permease)						
			<i>ytmJ</i>	3007	amino acid ABC transporter (binding protein)			
			<i>ytmK</i>	3006	amino acid ABC transporter (binding protein)			
			<i>ytmL</i>	3005	amino acid ABC transporter (permease)			
			<i>ytmM</i>	3005	amino acid ABC transporter (permease)			
			<i>ytnA</i>	3125	proline permease			
			<i>ytrE</i>	3118	ABC transporter (ATP-binding protein)			
			<i>ytrB</i>	3115	ABC transporter (ATP-binding protein)			
			<i>ytsC</i>	3111	ABC transporter (ATP-binding protein)			
			<i>ytsD</i>	3110	ABC transporter (permease)			
			<i>ytsB</i>	3108	multidrug resistance protein			
			<i>yubD</i>	3192	multidrug resistance protein			
			<i>yubG</i>	3188	Na ⁺ -transporting ATP synthase			
			<i>yufN</i>	3239	ABC transporter (lipoprotein)			
			<i>yufO</i>	3240	ABC transporter (ATP-binding protein)			
			<i>yufR</i>	3244	organic acid transport protein			
			<i>yufU</i>	3248	Na ⁺ /H ⁺ antiporter			
			<i>yufV</i>	3249	Na ⁺ /H ⁺ antiporter			
			<i>yugO</i>	3218	potassium channel protein			
			<i>yunJ</i>	3330	purine permease			
			<i>yunK</i>	3331	purine permease			
			<i>yurJ</i>	3345	multiple sugar ABC transporter (ATP-binding protein)			
			<i>yurM</i>	3348	sugar permease			
			<i>yurN</i>	3349	sugar permease			
			<i>yurO</i>	3350	multiple sugar-binding protein			
			<i>yurY</i>	3360	ABC transporter (ATP-binding protein)			
			<i>yusC</i>	3363	ABC transporter (ATP-binding protein)			
			<i>yusP</i>	3374	multidrug-efflux transporter			
			<i>yusV</i>	3379	iron(III) diclimate transport permease			
			<i>yutK</i>	3307	Na ⁺ /nucleoside cotransporter			
			<i>yuxJ</i>	3232	multidrug-efflux transporter			
			<i>yvaE</i>	3448	multidrug-efflux transporter			
			<i>yvbW</i>	3490	amino acid permease			
			<i>yvcC</i>	3579	ABC transporter (ATP-binding protein)			
			<i>yvcR</i>	3565	ABC transporter (ATP-binding protein)			
			<i>yvcS</i> </					

<i>flgK</i>	1700	flagellar hook protein	<i>coiX</i>	1251	spore coat protein (insoluble fraction)			SASP)	
<i>flgM</i>	3639	flagellar hook-associated protein 1 (HAP1)	<i>coiY</i>	1250	spore coat protein (insoluble fraction)		<i>sspE</i>	937	small acid-soluble spore protein (major γ -type SASP)
<i>flgN</i>	3637	flagellar hook-associated protein 3 (HAP3)	<i>coiZ</i>	1249	spore coat protein (insoluble fraction)				
<i>flgP</i>	3640	flagellin synthesis regulatory protein (anti-sigma factor [σ^H])	<i>csqA</i>	228	sporulation-specific SASP protein		<i>sspF</i>	53	small acid-soluble spore protein (minor α/β -type SASP)
<i>flhA</i>	1707	flagella-associated protein	<i>jag</i>	4213	SpolII-associated protein				
<i>flhB</i>	1706	flagella-associated protein	<i>kapB</i>	3230	activator of KinB in the initiation of sporulation		<i>usd</i>	3748	required for translation of <i>spoIIID</i>
<i>flhF</i>	1709	flagella-associated protein	<i>kapD</i>	3232	inhibitor of the KinA pathway to sporulation		<i>ynkT</i>	1495	sporulation protein σ^H -controlled
<i>flhO</i>	3746	flagellar basal-body rod protein	<i>kbaA</i>	159	activation of the KinB signaling pathway to sporulation		<i>ynkU</i>	1496	spore cortex membrane protein
<i>flhP</i>	3745	flagellar hook-basal body protein				<i>ynzH</i>	1901	spore coat protein	
<i>flhD</i>	3633	flagellar hook-associated protein 2 (HAP2)	<i>obg</i>	2853	GTP-binding protein involved in initiation of sporulation (SpoOA activation)		<i>yobW</i>	2083	membrane protein σ^H -controlled
<i>flhE</i>	1632	flagellar hook-basal body protein				<i>yagT</i>	2568	γ -glutamyl-L-alanine acid endopeptidase I	
<i>flhG</i>	1632	flagellar basal-body M-ring protein	<i>phrA</i>	1316	phosphatase (RapA) inhibitor (imported by Opp)		<i>yagQ</i>	2463	lipoprotein SpoII-Like
<i>flhH</i>	1694	flagellar motor switch protein	<i>phrC</i>	430	phosphatase (RapC) regulator / competence and sporulation stimulating factor (CSF)		<i>yraE</i>	2754	spore coat protein
<i>flhI</i>	1695	flagellar assembly protein				<i>yraF</i>	2752	spore coat protein	
<i>flhJ</i>	1695	flagellar-specific ATP synthase	<i>phrE</i>	2660	phosphatase (RapE) regulator		<i>yraG</i>	2752	spore coat protein
<i>flhK</i>	1697	flagellar protein required for formation of basal body	<i>phrF</i>	3846	phosphatase (RapF) regulator		<i>yraB</i>	2845	spore coat protein
<i>flhL</i>	1698	flagellar hook-length control	<i>phrG</i>	4141	phosphatase (RapG) regulator		<i>yraC</i>	2844	spore coat protein
<i>flhM</i>	1701	flagellar motor switch protein	<i>phrI</i>	548	phosphatase (RapI) regulator		<i>yraD</i>	2843	spore coat protein
<i>flhN</i>	1704	flagellar protein required for flagellar formation	<i>phrK</i>	2063	phosphatase (RapK) regulator		<i>yraE</i>	3161	spore coat protein
<i>flhO</i>	1705	flagellar protein required for flagellar formation	<i>rapA</i>	1315	response regulator aspartate phosphatase [SpoOF-P]		<i>yrgP</i>	3074	spore cortex protein
<i>flhP</i>	1706	flagellar protein required for flagellar formation				<i>yrgT</i>	3051	DNA translocase stage III sporulation protein	
<i>flhR</i>	1705	flagellar protein required for flagellar formation	<i>rapB</i>	3771	response regulator aspartate phosphatase [SpoOF-P]		<i>yrgA</i>	4208	DNA-binding protein SpoII-like
<i>flhS</i>	1702	flagellar motor switch protein	<i>rapC</i>	428	response regulator aspartate phosphatase				
<i>flhT</i>	1704	flagellar protein required for flagellar formation	<i>rapD</i>	3743	response regulator aspartate phosphatase		I.9	GERMINATION	23
<i>flhU</i>	1702	flagellar motor switch protein	<i>rapE</i>	2658	response regulator aspartate phosphatase		<i>gerAA</i>	3390	germination response to L-alanine
<i>flhV</i>	1702	flagellar motor switch protein	<i>rapF</i>	3845	response regulator aspartate phosphatase		<i>gerAB</i>	3391	germination response to L-alanine
<i>flhW</i>	1704	flagellar protein required for flagellar formation	<i>rapG</i>	4139	response regulator aspartate phosphatase		<i>gerAC</i>	3392	germination response to L-alanine
<i>hag</i>	3635	flagellin protein	<i>rapH</i>	750	response regulator aspartate phosphatase		<i>gerBA</i>	3688	germination response to the combination of glucose, fructose, L-asparagine, and KCl
<i>mcpA</i>	3207	methyl-accepting chemotaxis protein (glucose and α -methyl-glucoside)	<i>rapI</i>	547	response regulator aspartate phosphatase				
<i>mcpB</i>	3212	methyl-accepting chemotaxis protein (asparagine, glutamine and histidine)	<i>rapJ</i>	304	response regulator aspartate phosphatase		<i>gerBB</i>	3689	germination response to the combination of glucose, fructose, L-asparagine, and KCl
<i>mcpC</i>	1463	methyl-accepting chemotaxis protein (cysteine, proline, threonine, glycine, serine, lysine, valine and arginine)	<i>rapK</i>	2061	response regulator aspartate phosphatase		<i>gerBC</i>	3690	germination response to the combination of glucose, fructose, L-asparagine, and KCl
			<i>soj</i>	4206	antagonist of SinR		<i>gerCA</i>	2384	heptaprenyl diphosphate synthase component I (menaquinone biosynthesis)
							<i>gerCB</i>	2383	menaquinone biosynthesis methyltransferase (menaquinone biosynthesis)
<i>motA</i>	1435	motility protein (flagellar motor rotation)	<i>spB</i>	1461	spore maturation protein (spore core dehydratation)		<i>gerCC</i>	2382	heptaprenyl diphosphate synthase component II (menaquinone biosynthesis)
<i>motB</i>	1434	motility protein (flagellar motor rotation)	<i>spmA</i>	2423	spore maturation protein (spore core dehydratation)		<i>gerD</i>	159	germination response to L-alanine and to the combination of glucose, fructose, L-asparagine, and KCl
<i>tipA</i>	3209	methyl-accepting chemotaxis protein							
<i>tipB</i>	3205	methyl-accepting chemotaxis protein	<i>spmB</i>	2422	spore maturation protein (spore core dehydratation)		<i>gerKA</i>	420	germination response to the combination of glucose, fructose, L-asparagine, and KCl
<i>tipC</i>	374	methyl-accepting chemotaxis protein				<i>gerKB</i>	423	germination response to the combination of glucose, fructose, L-asparagine, and KCl	
<i>yfmS</i>	808	methyl-accepting chemotaxis protein	<i>spoOB</i>	2854	sporulation initiation phosphoprotein (part of phosphorelay: SpoOF-P->SpoOB-P->SpoOA-P)		<i>gerKC</i>	421	germination response to the combination of glucose, fructose, L-asparagine, and KCl
<i>ylhV</i>	1113	methyl-accepting chemotaxis protein	<i>spoOE</i>	1430	negative sporulation regulatory phosphatase [SpoOA-P]		<i>gerM</i>	2902	germination (cortex hydrolysis) and sporulation (stage II, multiple polar septa)
<i>ylhG</i>	1679	flagellar biosynthetic protein	<i>spoOI</i>	4206	chromosome positioning near the pole and transport through the polar septum / antagonist of Soj		<i>gpr</i>	2635	spore protease (degradation of SASPs)
<i>ylhX</i>	1699	flagellar hook assembly protein	<i>spollAA</i>	2444	anti-anti-sigma factor (of SpoIIAB)		<i>sleB</i>	2399	spore cortex-lytic enzyme
<i>yohA</i>	2030	flagellar biosynthesis switch protein	<i>spollAB</i>	2444	anti-sigma factor (σ^H [SpoIIAC]) and serine kinase [SpoII-AA]		<i>yfkQ</i>	850	spore germination response
<i>yohD</i>	3043	flagellar motor apparatus	<i>spollB</i>	2864	endospore development (oligosporogenous mutation)		<i>yfkR</i>	848	spore germination protein
<i>yohE</i>	3042	flagellar motor apparatus	<i>spollD</i>	3777	required for complete dissolution of the asymmetric septum		<i>yktT</i>	847	spore germination protein
<i>yoaQ</i>	3457	transmembrane receptor taxis protein	<i>spollE</i>	71	serine phosphatase [SpoIIAA-P] (σ^H activation) / asymmetric septum formation		<i>ykvT</i>	1448	spore cortex-lytic enzyme
<i>yyvC</i>	3634	flagellar protein				<i>yndD</i>	1907	spore germination protein	
<i>yyvF</i>	3640	flagellar protein	<i>spollIA</i>	1603	protease (processing of pro- σ^H to active σ^H)		<i>yndF</i>	1908	spore germination protein
<i>yyvG</i>	3639	flagellar protein	<i>spollIB</i>	2536	mutants block sporulation after engulfment				
<i>yozB</i>	3609	flagellin	<i>spollIAD</i>	2535	mutants block sporulation after engulfment				
			<i>spollIAC</i>	2535	mutants block sporulation after engulfment				
			<i>spollIAD</i>	2535	mutants block sporulation after engulfment				
			<i>spollIAD</i>	2535	mutants block sporulation after engulfment				
			<i>spollIAF</i>	2534	mutants block sporulation after engulfment				
			<i>spollIAF</i>	2533	mutants block sporulation after engulfment				
			<i>spollIAH</i>	2532	mutants block sporulation after engulfment				
			<i>spollIE</i>	1752	DNA translocase required for chromosome partitioning through the septum into the forespore				
			<i>spollIU</i>	4214	essential for σ^H activity at stage III				
			<i>spollIM</i>	2450	required for dissolution of the septal cell wall				
			<i>spollIP</i>	2634	required for dissolution of the septal cell wall				
			<i>spollIQ</i>	3780	required for completion of engulfment				
			<i>spollIR</i>	3794	required for processing of pro- σ^H				
			<i>spollISA</i>	1349	lethal when synthesized during vegetative growth in the absence of SpoIIB				
			<i>spollISB</i>	1348	disruption blocks sporulation after septum formation				
			<i>spolVA</i>	2387	required for proper spore cortex formation and coat assembly				
			<i>spolVB</i>	2520	intercompartmental signalling of pro- σ^H processing / activation in the mother-cell				
			<i>spolVCA</i>	2654	site-specific DNA recombinase required for creating the <i>sigK</i> gene (excision of the <i>skin</i> element)				
			<i>spolVFA</i>	2857	inhibitor of SpoIVFB				
			<i>spolVFB</i>	2856	protease (processing of pro- σ^H to active σ^H)				
			<i>spolVAA</i>	2443	mutants lead to the production of immature spores				
			<i>spolVAB</i>	2442	mutants lead to the production of immature spores				
			<i>spolVAC</i>	2441	mutants lead to the production of immature spores				
			<i>spolVAD</i>	2441	mutants lead to the production of immature spores				
			<i>spolVAE</i>	2440	mutants lead to the production of immature spores				
			<i>spolVAF</i>	2439	mutants lead to the production of immature spores				
			<i>spolVB</i>	2829	involved in spore cortex synthesis				
			<i>spolVC</i>	60	thermosensitive mutant blocks spore coat formation				
			<i>spolVE</i>	1590	required for spore cortex synthesis				
			<i>spolVFA</i>	1744	dipicolinate synthase subunit A				
			<i>spolVFB</i>	1745	dipicolinate synthase subunit B				
			<i>spolVG</i>	56	required for spore cortex synthesis				
			<i>spolVID</i>	2872	required for assembly of the spore coat				
			<i>spolVK</i>	1873	disruption leads to the production of immature spores				
			<i>spolVM</i>	1655	required for normal spore cortex and coat synthesis				
			<i>spolVR</i>	1015	involved in spore cortex synthesis				
			<i>spolVS</i>	1769	required for dehydratation of the spore core and assembly of the coat				
			<i>spaA</i>	3892	spore coat polysaccharide synthesis				
			<i>spaB</i>	3891	spore coat polysaccharide synthesis				
			<i>spaC</i>	3890	spore coat polysaccharide synthesis				
			<i>spaD</i>	3889	spore coat polysaccharide synthesis				
			<i>spaE</i>	3888	spore coat polysaccharide synthesis				
			<i>spaF</i>	3887	spore coat polysaccharide synthesis				
			<i>spaG</i>	3886	spore coat polysaccharide synthesis				
			<i>spaH</i>	3885	spore coat polysaccharide synthesis				
			<i>spaI</i>	3884	spore coat polysaccharide synthesis				
			<i>spaK</i>	3883	spore coat polysaccharide synthesis				
			<i>spaP</i>	3025	small acid-soluble spore protein (major α -type SASP)				
			<i>sspB</i>	1050	small acid-soluble spore protein (major β -type SASP)				
			<i>sspC</i>	2155	small acid-soluble spore protein (minor α/β -type SASP)				
			<i>sspD</i>	1413	small acid-soluble spore protein (minor α/β -type SASP)				

I.6

PROTEIN SECRETION

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<i>csaA</i>	2079	chaperonin involved in protein secretion	<i>spollIGA</i>	1603	protease (processing of pro- σ^H to active σ^H)				
<i>flh</i>	1672	signal recognition particle	<i>spollIAB</i>	2537	mutants block sporulation after engulfment				
<i>flh</i>	1672	signal recognition particle	<i>spollIAB</i>	2536	mutants block sporulation after engulfment				
<i>lsp</i>	1610	signal peptidase II	<i>spollIAC</i>	2535	mutants block sporulation after engulfment				
<i>ylhA</i>	3652	signal peptidase I	<i>spollIAD</i>	2535	mutants block sporulation after engulfment				
<i>prsa</i>	1071	protein secretion (post-translocation chaperonin)	<i>spollIAD</i>	2535	mutants block sporulation after engulfment				
<i>secA</i>	3630	preprotein translocase subunit	<i>spollIAF</i>	2534	mutants block sporulation after engulfment				
<i>secE</i>	118	preprotein translocase subunit	<i>spollIAF</i>	2533	mutants block sporulation after engulfment				
<i>secF</i>	2829	protein-export membrane protein (product also similar to SecD of <i>E. coli</i>)	<i>spollIAH</i>	2532	mutants block sporulation after engulfment				
			<i>spollIE</i>	1752	DNA translocase required for chromosome partitioning through the septum into the forespore				
<i>secY</i>	145	preprotein translocase subunit							
<i>spS</i>	2432	signal peptidase I	<i>spollIU</i>	4214	essential for σ^H activity at stage III				
<i>spT</i>	1511	signal peptidase I	<i>spollIM</i>	2450	required for dissolution of the septal cell wall				
<i>spU</i>	454	signal peptidase I	<i>spollIP</i>	2634	required for dissolution of the septal cell wall				
<i>spW</i>	1122	signal peptidase I	<i>spollIQ</i>	3780	required for completion of engulfment				
<i>spX</i>	254	signal peptidase II	<i>spollIR</i>	3794	required for processing of pro- σ^H				
<i>yaaT</i>	81	signal peptidase II	<i>spollISA</i>	1349	lethal when synthesized during vegetative growth in the absence of SpoIIB				
<i>yacD</i>	42	general secretion PrsA homologue							
<i>yobE</i>	2057	general secretion pathway protein	<i>spollISB</i>	1348	disruption blocks sporulation after septum formation				

I.7

CELL DIVISION

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<i>divIB</i>	1593	σ^H -division initiation protein (septum formation)	<i>spolVA</i>	2387	required for proper spore cortex formation and coat assembly				
<i>divIC</i>	69	cell-division initiation protein (septum formation)	<i>spolVB</i>	2520	intercompartmental signalling of pro- σ^H processing / activation in the mother-cell				
<i>divIV</i>	1612	cell-division initiation protein (septum placement)							
<i>ftsA</i>	1596	cell-division protein (septum formation)	<i>spolVCA</i>	2654	site-specific DNA recombinase required for creating the <i>sigK</i> gene (excision of the <i>skin</i> element)				
<i>ftsE</i>	3625	cell-division ATP-binding protein	<i>spolVFA</i>	2857	inhibitor of SpoIVFB				
<i>ftsH</i>	77	cell-division protein / general stress protein (class III heat-shock)	<i>spolVFB</i>	2856	protease (processing of pro- σ^H to active σ^H)				
<i>ftsL</i>	1581	cell-division protein (septum formation)	<i>spolVAA</i>	2443	mutants lead to the production of immature spores				
<i>ftsX</i>	3624	cell-division protein	<i>spolVAB</i>	2442	mutants lead to the production of immature spores				
<i>gid</i>	1685	glucose-inhibited division protein	<i>spolVAC</i>	2441	mutants lead to the production of immature spores				
<i>gidA</i>	4211	glucose-inhibited division protein	<i>spolVAD</i>	2441	mutants lead to the production of immature spores				
<i>gidB</i>	4209	glucose-inhibited division protein	<i>spolVAE</i>	2440	mutants lead to the production of immature spores				
<i>maf</i>	2862	septum formation	<i>spolVAF</i>	2439	mutants lead to the production of immature spores				
<i>minC</i>	2859	cell-division inhibitor (septum placement)							
<i>minD</i>	2858	cell-division inhibitor (septum placement) (ATPase activator of MinC)	<i>spolVB</i>	2829	involved in spore cortex synthesis				
<i>yacA</i>	75	cell-cycle protein	<i>spolVC</i>	60	thermosensitive mutant blocks spore coat formation				
<i>yfhF</i>	925	cell-division inhibitor	<i>spolVE</i>	1590	required for spore cortex synthesis				
<i>yobB</i>	3214	cell-division protein FtsH homologue	<i>spolVFA</i>	1744	dipicolinate synthase subunit A				
<i>yolC</i>	1552	cell-division protein	<i>spolVFB</i>	1745	dipicolinate synthase subunit B				
<i>ylmH</i>	1611	cell-division protein	<i>spolVG</i>	56	required for spore cortex synthesis				
<i>ywcF</i>	3912	cell-division protein	<i>spolVID</i>	2872	required for assembly of the spore coat				

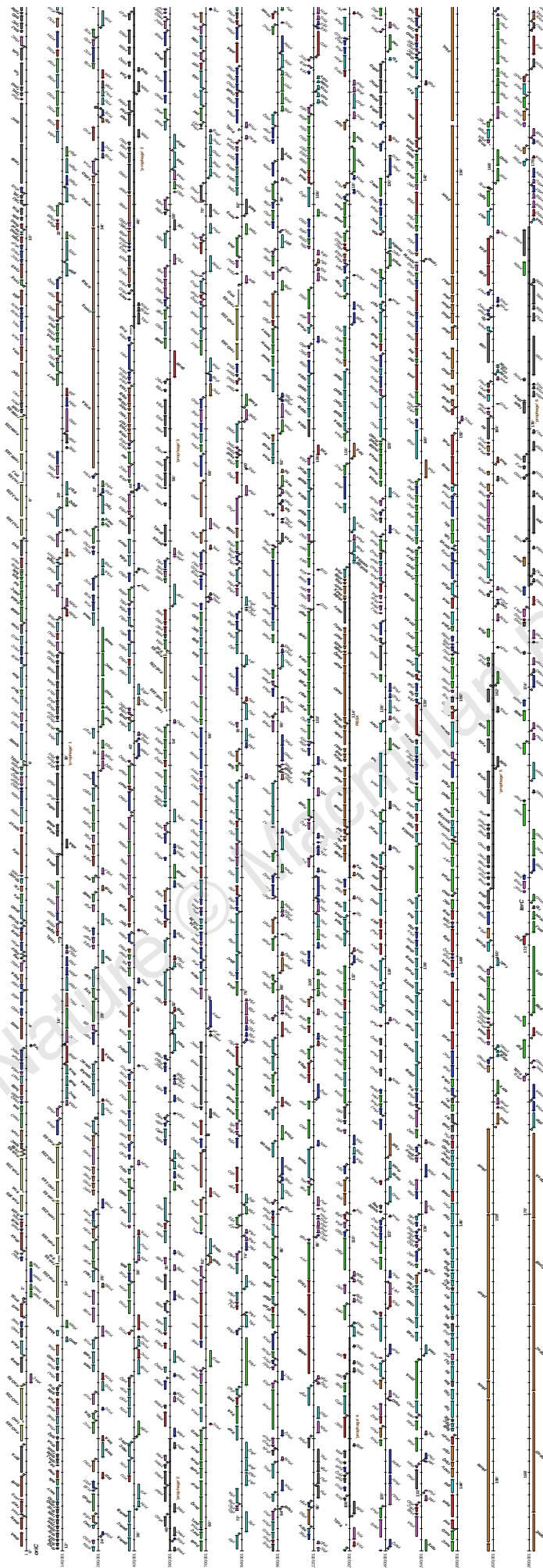
I.8

SPORULATION

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<i>bofA</i>	30	inhibitor of the pro- σ^H processing machinery	<i>spolVK</i>	1873	disruption leads to the production of immature spores				
<i>bofC</i>	2837	forespore regulator of the σ^H checkpoint	<i>spolVM</i>	1655	required for normal spore cortex and coat synthesis				

glsH	4033	β-glucosidase (cellulose degradation)	yjmA	1300	glucuronate isomerase	mmgD	2510	citrate synthase III
bgIS	4031	endo-1-3-1,4 glucanase (lichenan degradation)	yjmD	1304	sorbitol dehydrogenase	odhA	2111	2-oxoglutarate dehydrogenase (E1 subunit)
crn	3569	catabolite repression HPr-like protein	yjme	1305	o-mannanotase hydrolase	odhB	2108	2-oxoglutarate dehydrogenase (dihydrolipoamide transsuccinylase, E2 subunit)
csrA	3635	carbon storage regulator	yjmf	1306	2-deoxy-o-glucanate 3-dehydrogenase	sdhA	2907	succinate dehydrogenase (flavoprotein subunit)
frbB	1508	fructose 1-phosphate kinase	yjml	1311	altronate hydrolase	sdhB	2905	succinate dehydrogenase (iron-sulphur protein)
galE	3990	UDP-glucose 4-epimerase (galactose metabolism)	yjmc	1356	dolichol phosphate mannose synthase	sdhC	2908	succinate dehydrogenase (cytochrome b ₅₅₈ subunit)
galK	3921	galactokinase (galactose metabolism)	yjfc	1366	chloromuconate cycloisomerase	sucC	1680	succinyl-CoA synthetase (β subunit)
galT	3919	galactanase 1-phosphate uridylyltransferase (galactose metabolism)	yjkt	1367	polysugar degrading enzyme	sucD	1681	succinyl-CoA synthetase (α subunit)
gdh	445	glucose 1-dehydrogenase	yjkrV	1427	ribulose-bisphosphate carboxylase	yjmc	1303	malate dehydrogenase
glcK	2571	glucose kinase	yjkc	1537	myo-inositol-1 or 4-monomorphosphate	yqkI	2452	malate dehydrogenase
glgA	3167	starting (bacterial glycogen) synthase (glycogen biosynthesis)	yjku	1477	glucose 1-dehydrogenase	ytsI	2930	malate dehydrogenase
glgB	3171	1,4-glucan branching enzyme (glycogen biosynthesis)	yjko	1442	glucose 1-dehydrogenase	ywkA	3801	malate dehydrogenase
glgC	3169	glucose-1-phosphate adenylyltransferase (glycogen biosynthesis)	yjor	1653	ribulose-5-phosphate 3-epimerase	Il.2	METABOLISM OF AMINO ACIDS AND RELATED MOLECULES	2
glgD	3168	required for glycogen biosynthesis	yjY	1741	deacetylase	ald	3277	L-alanine dehydrogenase
glpE	3165	glucose phosphorylase (glycogen metabolism)	yngE	1951	propionyl-CoA carboxylase	ampS	1516	aminopeptidase
glpD	1004	glycerol-3-phosphate dehydrogenase (glycerol utilization)	yocA	2023	xylulokinase	ansA	2456	L-asparaginase
glpK	1003	glycerol kinase (glycerol utilization)	yodA	2024	phosphoglycerate dehydrogenase	ansB	2455	L-aspartase
glvA	6-phospho-glucosidase (arbutin fermentation)	yoeA	2025	formate dehydrogenase	aprE	1105	extracellular alkaline serine protease (subtilisin E)	
gnrK	4113	glucose kinase (gluconate utilization)	yol	2031	4-hydroxyphenylacetate-3-hydroxylase	aprX	1862	intracellular alkaline serine protease
gnrZ	4116	6-phosphogluconate dehydrogenase (gluconate utilization)	yogA	2007	alcohol dehydrogenase	argB	1197	N-acetylglutamate 5-phosphotransferase (arginine biosynthesis)
gpsA	2389	NAD(P)H-dependent glycerol-3-phosphate dehydrogenase	yogB	2507	phosphoenolpyruvate mutase	argC	1195	N-acetylglutamate γ-semialdehyde dehydrogenase (arginine biosynthesis)
gutB	667	sorbitol dehydrogenase	yoid	2488	apocyclic-CoA carboxylase	argD	1198	N-acetylornithine aminotransferase (arginine biosynthesis)
iolB	4082	myo-inositol catabolism	yriH	2778	methylethyltransferase	argE	2142	N-acetylornithine deacetylase (arginine biosynthesis)
iolC	4081	myo-inositol catabolism	yriH	2768	cyclodextrin metabolism	argF	1203	ornithine carbamoyltransferase (arginine biosynthesis)
iolD	4080	myo-inositol catabolism	yrgP	2742	sugar-phosphate dehydrogenase	argG	3013	ornithine carbamoyltransferase (arginine biosynthesis)
iolE	4078	myo-inositol catabolism	yrdC	2950	endo-1,4-glucanase	argH	3012	argininosuccinate lyase (arginine biosynthesis)
iolG	4076	myo-inositol 2-dehydrogenase (inositol catabolism)	yscD	2932	glycolate oxidase subunit	argI	1196	ornithine acetyltransferase / amino-acid acetyltransferase (arginine biosynthesis)
iolH	4075	myo-inositol catabolism	yscF	2934	glycolate oxidase subunit	aroA	3046	3-deoxy-α-arabino-heptulosonate 7-phosphate synthase / chorismate mutase-isozyme 3 (shikimate pathway)
iolI	4074	myo-inositol catabolism	ytdE	2969	plant metabolite dehydrogenase	aroB	2378	3-dehydroquinate synthase (shikimate pathway)
kdgA	2323	deoxyphosphogluconate aldolase (pectin utilization)	ytdA	3155	NDP-sugar dehydrogenase	aroC	2413	3-dehydroquinate dehydratase (shikimate pathway)
kdgK	2324	2-keto-3-deoxygluconate kinase (pectin utilization)	ytdB	3156	NDP-sugar epimerase	aroD	2645	shikimate 5-dehydrogenase (shikimate pathway)
kduD	2326	2-keto-3-deoxygluconate oxidoreductase (pectin utilization)	ytdC	3157	NDP-sugar epimerase	aroE	2368	5-enolpyruvylshikimate-3-phosphate synthase (shikimate pathway)
kdul	2325	5-keto-4-deoxyuronate isomerase (pectin utilization)	ytdE	3158	NDP-sugar epimerase	aroF	2380	chorismate synthase (shikimate pathway)
lacA	3504	β-galactosidase	ytdF	3159	NDP-sugar epimerase	aroH	2377	chorismate mutase (isozymes 1 and 2) (aromatic amino acids biosynthesis)
lctE	329	γ-lactate dehydrogenase	ytdG	3160	NDP-sugar epimerase	arol	340	shikimate kinase (shikimate pathway)
ldhE	3952	6-phospho-β-glucosidase	ytdH	3161	NDP-sugar epimerase	asd	1745	aspartate-semialdehyde dehydrogenase
ldhD	782	hydrolytic enzyme	ytdI	3162	NDP-sugar epimerase	ask	2910	aspartokinase II attenuator
melA	3100	α-D-galactoside galactohydrolase	ytdJ	3163	NDP-sugar epimerase	asb	3127	asparagine synthetase
mtiD	4051	mannotol-1-phosphate dehydrogenase	ytdK	3164	NDP-sugar epimerase	asnH	4098	asparagine synthetase
nagA	3594	N-acetylglucosamine-6-phosphate deacetylase (N-acetylglucosamine utilization)	ytdL	3165	NDP-sugar epimerase	aspB	2348	aspartate aminotransferase
nagB	3596	N-acetylglucosamine-6-phosphate isomerase (N-acetylglucosamine utilization)	ytdM	3166	NDP-sugar epimerase	bcsA	2317	naringenin-chalcone synthase (phenylalanine metabolism)
narQ	3773	required for formate dehydrogenase activity	ytdN	3167	NDP-sugar epimerase	bfmBAA	2499	branched-chain α-keto acid dehydrogenase E1 (2-oxoisovalerate dehydrogenase α subunit)
pelB	828	pectate lyase	ytdO	3502	arabinogalactan endo-1,4-galactosidase	bfmBAB	2498	branched-chain α-keto acid dehydrogenase E1 (2-oxoisovalerate dehydrogenase β subunit)
pelB	2034	pectate lyase	ytdP	3493	hydrolase	bfmBB	2497	branched-chain α-keto acid dehydrogenase E2 subunit (lipoamide acyltransferase)
pps	3698	mannose-6-phosphate isomerase	ytdQ	3495	acolate oxidase	btdD	2718	spermine / spermidine acetyltransferase
pta	3865	phosphotransacetylase	yjvN	3427	plant-metabolite dehydrogenase	bpr	1599	bacillopeptidase F
ptsH	1459	histidine-containing phosphocarrier protein of the phosphotransferase system (PTS) (HPr protein)	yjvC	3615	pyruvate, water dikinase	cad	1535	lysine decarboxylase
ptsK	3701	ribokinase (ribose metabolism)	yjvE	3592	phosphoglycolate phosphatase	carA	1199	carbamoyl-phosphate transferase-arginine (sub-unit A) (arginine biosynthesis)
sacA	3902	sucrase-6-phosphate hydrolase	yjvF	3591	O-acetyltransferase	carB	1200	carbamoyl-phosphate transferase-arginine (sub-unit B) (arginine biosynthesis)
sacB	3535	levansucrase	yjvP	3590	pectate lyase	ctpA	2133	carboxy-terminal processing protease
sacC	2759	levanase	yjvH	3664	UDP-N-acetylglucosamine 2-epimerase	cysE	113	serine acetyltransferase (cysteine biosynthesis)
sacX	3941	negative regulatory protein of SacY	yjvI	3895	aldehyde dehydrogenase	cysH	1630	phosphoadenosine phosphosulfate reductase (cysteine biosynthesis)
treA	891	trehalose-6-phosphate hydrolase	yjvJ	3872	glucose 1-dehydrogenase	cysK	82	cysteine synthetase A (cysteine biosynthesis)
xxa	251	β-xylosidase / α-L-arabinosidase (xylan degradation)	yjvK	3850	glycerol-inducible protein	dal	517	D-alanine racemase
xyIA	1891	xyllose isomerase (xyllose metabolism)	yjvL	3730	NDP-sugar dehydrogenase	dapA	1748	dihydropicolinate synthase (diaminopimelate / lysine biosynthesis)
xyIB	1893	xylulose kinase (xyllose metabolism)	yjvD	4091	glucose 1-dehydrogenase	dapB	2359	dihydropicolinate reductase (diaminopimelate / lysine biosynthesis)
xynA	2054	endo-1,4-xylanase (xylan degradation)	yjvE	4040	endo-1,5-L-arabinosidase	dapG	1747	aspartokinase I (α and β subunits)
xynB	1888	xylan β-1,4-xylosidase (xylan degradation)	yjvF	4000	gluconate 5-dehydrogenase	def	1646	polypeptide deformylase
xynD	1945	endo-1,4-xylanase (xylan degradation)	yjvG	4107	glucose 1-dehydrogenase	epo	3939	minor extracellular serine protease
ybaN	161	polysaccharide deacetylase	yjvH	4202	formate dehydrogenase	gmsD	200	L-glutamine-0-fructose-6-phosphate amidotransferase
ybbD	188	β-hexosaminidase	yjvI	4196	galactoside acetyltransferase	glnA	1878	glutamine synthetase
ybcM	213	glucosamine-fructose-6-phosphate aminotransferase	yjvJ	4136	formaldehyde dehydrogenase	gltA	2014	glutamate synthase (large subunit) (glutamate biosynthesis)
ybtT	258	glucosamine-6-phosphate isomerase	Il.12	MAIN GLYCOLYTIC PATHWAYS	gltB	2009	glutamate synthase (small subunit) (glutamate biosynthesis)	
yctB	268	5-dehydro-D-deoxyglucarate dehydratase	eno2	3477	enolase (glycolysis)	gltC	3789	serine hydroxymethyltransferase (glycine / serine / threonine metabolism)
yctD	269	acetylacide dehydrogenase	tbaA	3808	fructose-1,6-bisphosphate aldolase (glycolysis)	hisA	3584	phosphoribosylformimino-5-aminoimidazole carboxamide ribotide isomerase (histidine biosynthesis)
ycbF	272	glutamate dehydratase	tkt	4127	fructose-1,6-bisphosphatase (gluconeogenesis)	hisB	3585	imidazoleglycerol-phosphate dehydratase (histidine biosynthesis)
ycdF	305	glucose 1-dehydrogenase	gap	3482	glyceraldehyde 3-phosphate dehydrogenase (glycolysis)	hisC	2371	glutathionyl-phosphate aminotransferase (histidine biosynthesis) / tyrosine and phenylalanine aminotransferase
ycdG	306	oligo-1,6-glucosidase	gapB	2967	glyceraldehyde 3-phosphate dehydrogenase (glycolysis)	hisD	3587	histidinol dehydrogenase (histidine biosynthesis)
ycgS	352	aromatic hydrocarbon catabolism	iolI	4073	fructose-1,6-bisphosphate aldolase (glycolysis)	hisF	3583	HisF cyclase-like protein (synthesis of D-erythroidiamidazole glycerol phosphate)
yckE	370	β-glucosidase	pckA	3129	phosphoenolpyruvate carboxykinase	hisG	3587	ATP phosphoribosyltransferase (histidine biosynthesis)
yckG	375	D-arabino-3-hexulose 6-phosphate formaldehyde lyase	pdhA	1528	pyruvate dehydrogenase (E1 α subunit)	hisH	3585	amidotransferase (histidine biosynthesis)
ycsN	466	aryl-alcohol dehydrogenase	pdhB	1529	pyruvate dehydrogenase (E1 β subunit)	hisI	3583	phosphoribosyl-AMP cyclohydrolase / phosphoribosyl-ATP pyrophosphohydrolase (histidine biosynthesis)
ydaD	471	alcohol dehydrogenase	pdhC	1530	pyruvate dehydrogenase (dihydrolipoamide acyltransferase E2 subunit)	hom	3315	homoserine dehydrogenase (threonine / methionine biosynthesis)
ydaF	473	acetyltransferase	pdhD	1531	pyruvate dehydrogenase / 2-oxoglutarate dehydrogenase (dihydrolipoamide dehydrogenase E3 subunit)	hutG	4045	formingonlutamate hydrolase (histidine utilization)
ydaM	482	cellulose synthase	pkf	2987	6-phosphofructokinase (glycolysis)	hutH	4041	histidase (histidine utilization)
ydaP	488	reticuline oxidase	pgi	3221	glucose-6-phosphate isomerase (glycolysis)	hutI	4044	imidazole-5-propionate hydrolase (histidine utilization)
ydhP	628	β-glucosidase	pgk	3480	phosphoglycerate kinase (glycolysis)	hutU	4042	urocanase (histidine utilization)
ydhR	631	fructokinase	pgm	3478	phosphoglycerate mutase (glycolysis)	ilvA	2896	threonine dehydratase (isoleucine biosynthesis)
ydhS	632	mannose-6-phosphate isomerase	pvcA	1554	pyruvate carboxylase	ilvB	2896	acetoactolactate synthase (large subunit) (valine / isoleucine biosynthesis)
ydhT	632	mannan endo-1,4-mannosidase	pykA	2986	pyruvate kinase (glycolysis)	ilvC	2894	keto-acid reductoisomerase (valine / isoleucine biosynthesis)
ydjE	670	fructokinase	tkt	1919	transketolase (pentose phosphate)	ilvD	2302	dihydroxy-acid dehydratase (valine / isoleucine biosynthesis)
yjdl	679	L-iditol 2-dehydrogenase	tpi	3479	triose phosphate isomerase (glycolysis)	ilvN	2894	acetoactolactate synthase (small subunit)
yjdp	682	arylesterase	ybtT	198	phosphoglucomutase (glycolysis)			
yecA	688	methionine dehydrogenase regulation	ydeA	558	glyceraldehyde 3-phosphate dehydrogenase (glycolysis)			
yeyY	774	rhannogalacturonan acetyltransferase	yqrC	1109	phosphoglycerate mutase (glycolysis)			
yeyZ	774	β-galactosidase	yqrD	2651	6-phosphogluconate dehydrogenase (pentose phosphate)			
yfhM	829	epoxide hydrolase	yqjV	2501	dihydrolipoamide dehydrogenase			
yfhR	937	glucose 1-dehydrogenase	yqjL	2481	6-phosphogluconate dehydrogenase (pentose phosphate)			
yfS	969	polysaccharide deacetylase	yqjI	2478	glucose-6-phosphate 1-dehydrogenase (pentose phosphate)			
yfmT	807	benzaldehyde dehydrogenase	ywjH	3807	transaldolase (pentose phosphate)			
yfhH	798	glucose 1-phosphate cytidylyltransferase	ywjF	3791	ribose 5-phosphate epimerase (pentose phosphate)			
ygaK	958	reticuline oxidase						
yhcW	997	phosphoglycolate phosphatase						
yhdF	1022	glucose 1-dehydrogenase						
yhdN	1030	aldo / keto reductase						
yheN	1041	endo-1,4-xylanase						
yhiE	1095	glucanase						
yhxB	1006	phosphomannomutase	Il.13	TCA CYCLE				
yhxC	1115	alcohol dehydrogenase	citA	1021	citrate synthase I			
yhxD	1116	alcohol dehydrogenase	citB	1926	aconitate hydratase			
yisS	1164	myo-inositol 2-dehydrogenase	citC	2980	isocitrate dehydrogenase			
yitS	1175	mandelate racemase	citG	3389	fumarate hydratase			
yitY	1192	L-gulonolactone oxydase	citH	2979	malate dehydrogenase			
yidE	1274	mannose-6-phosphate isomerase	citZ	2891	citrate synthase II			
yieA	1281	endo-1,4-xylanase	malS	3058	malate dehydrogenase			
yjgC	1285	formate dehydrogenase						



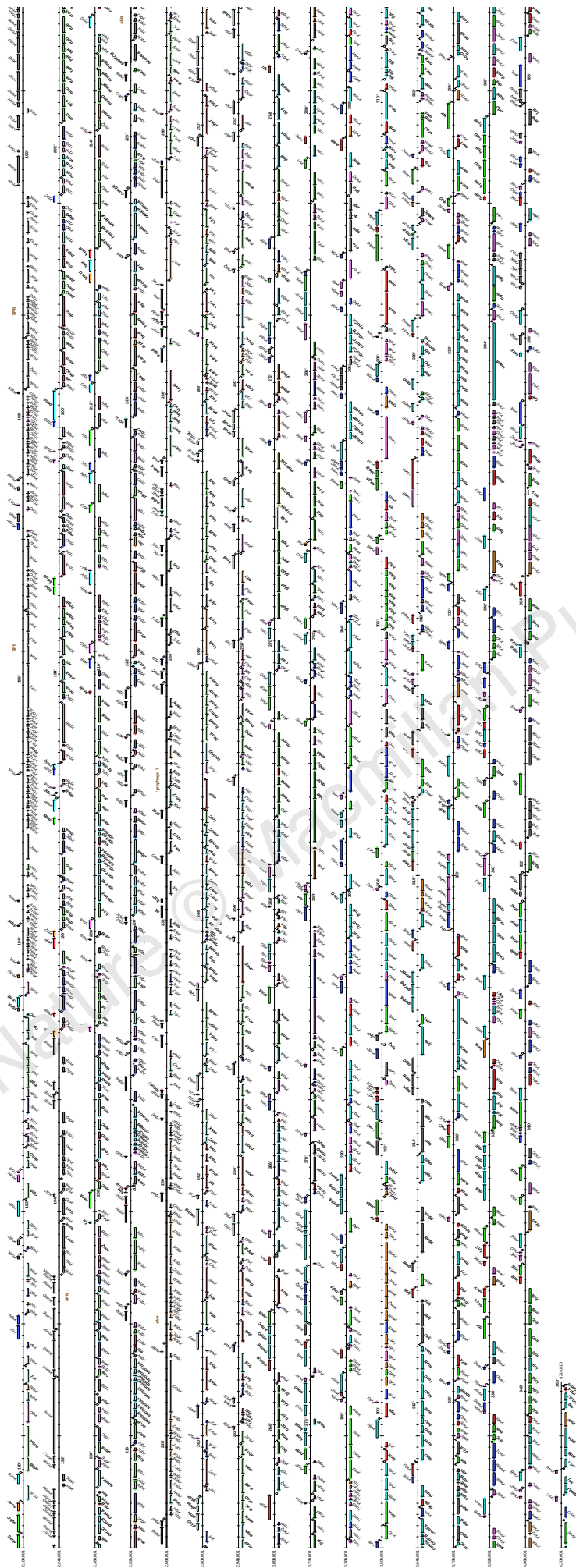


Table 1. (continuation) Functional classification of the *Bacillus subtilis* protein-coding genes.

		(valine/isoleucine biosynthesis)	yqjE	2486	tripeptidase	xpt	2319	xanthine phosphoribosyltransferase (purine biosynthesis)
ioIA	4083	methyilmalonate-semialdehyde dehydrogenase (valine metabolism)	yqjN	2475	amino acid degradation	yaaF	23	deoxypurine kinase subunit
ipi	1189	intracellular proteinase inhibitor	yqjR	2472	pyrroline-5-carboxylate reductase	yaaG	24	deoxypurine kinase subunit
ispA	1386	major intracellular serine protease	yrbE	2839	opine catabolism	yabR	70	polyribonucleotide nucleotidyltransferase
kbl	1771	2-amino-3-ketobutyrate CoA ligase	yrbF	2786	cysteine synthase	yera	713	adenine deaminase
leuA	2893	2-isopropylmalate synthase (leucine biosynthesis)	yrbP	2785	cystathionine γ -synthase	yfkm	859	2',3'-cyclic-nucleotide 2'-phosphodiesterase
leuB	2891	3-isopropylmalate dehydrogenase (leucine biosynthesis)	yrbY	2768	dihydrodipicolinate reductase	yhaM	1069	CMP-binding factor
leuC	2890	3-isopropylmalate dehydratase (large subunit) (leucine biosynthesis)	yrcP	2738	glutamate racemase	yhcR	991	5'-nucleotidase
leuD	2889	3-isopropylmalate dehydratase (small subunit) (leucine biosynthesis)	yrrN	2794	protease	yirY	1144	DNA exonuclease
lysA	2437	diaminopimelate decarboxylase (lysine biosynthesis)	yrrO	2793	protease	yjbM	1236	GTP pyrophosphokinase
lysC	2910	aspartokinase II (α and β subunits) (diaminopimelate/lysine biosynthesis)	ysnE	2897	acetyltransferase	yjbP	1240	diadenosine tetraphosphatase
metB	2305	homoserine O-succinyltransferase (methionine biosynthesis)	ytdD	3146	N-acetylaminic acid racemase	ykbE	1377	formyltetrahydrofolate deformylase
metC	1385	cobalamin-independent methionine synthase (methionine biosynthesis)	ytkP	3066	cysteine synthase	ykbB	1565	IMP dehydrogenase
metK	3128	S-adenosylmethionine synthetase	yubC	3193	cysteine dioxygenase	yloD	1641	guanylate kinase
mpr	245	extracellular metalloprotease	yugH	3226	aspartate aminotransferase	ymaA	1868	ribonucleoprotein
nasB	362	assimilatory nitrate reductase (electron transfer subunit)	yurG	3341	aspartate aminotransferase	ynbC	1895	micrococcal nuclease
nasC	360	assimilatory nitrate reductase (catalytic subunit)	yurH	3343	N-carbamyl-L-amino acid amidohydrolase	yncF	1899	deoxyuridine 5'-triphosphate pyrophosphatase
nasD	358	assimilatory nitrite reductase (subunit)	yurL	3347	opine catabolism	yosR	2165	ribonucleoside-diphosphate reductase (α subunit)
nasE	355	assimilatory nitrite reductase (subunit)	yurP	3351	opine catabolism	yosO	2164	ribonucleoside-diphosphate reductase (α subunit)
nprB	1186	extracellular neutral protease B	yurR	3353	opine catabolism	yosP	2161	ribonucleoside-diphosphate reductase (β subunit)
nprE	1541	extracellular neutral metalloprotease	yurT	3354	methyglyoxalase	yosS	2159	deoxyuridine 5'-triphosphate nucleotidohydrolase
nrgB	3757	nitrogen-regulated PII-like protein	yusH	3366	glycine cleavage system protein H	yprD	2395	ribosomal protein S1 homologue
patA	1472	aminotransferase	yusM	3373	proline dehydrogenase	yqjB	2528	exodeoxyribonuclease VII (large subunit)
patB	3228	aminotransferase	yusX	3381	oligoendopeptidase	yqjC	2526	exodeoxyribonuclease VII (small subunit)
pepT	3994	peptidase T	yutL	3306	diaminopimelate epimerase	yrdF	2730	ribonuclease inhibitor
pheA	2851	phenylalanine dehydratase (phenylalanine biosynthesis)	yuxL	3312	acylaminoacyl-peptidase	yrrU	2787	purine nucleoside phosphorylase
pheB	2852	chorismate mutase (phenylalanine biosynthesis)	yvaA	3454	carboxylesterase	yurD	3302	GMP reductase
proA	1379	γ -glutamyl phosphate reductase (proline biosynthesis)	yvdD	3516	serine O-acetyltransferase	yunH	3328	uracilase
proB	1378	γ -glutamyl kinase (proline biosynthesis)	yvbB	3623	carboxy-terminal processing protease	yunL	3332	uricase
proH	2017	involved in proline biosynthesis (salt-inducible)	ywaA	3956	branched-chain amino acid aminotransferase	yurL	3343	ribonuclease
proJ	2016	glutamate 5-kinase (proline biosynthesis)	ywaD	3947	aminopeptidase	ywaC	3949	GTP-pyrophosphokinase
racX	3533	amino acid racemase	yweB	3881	glutamate dehydrogenase	il4	77	METABOLISM OF LIPIDS
rocA	3879	pyrroline-5-carboxylate dehydrogenase (arginine and ornithine utilization)	ywgG	3868	aspartate aminotransferase	accB	2988	acetyl-CoA carboxylase (α subunit) (long-chain fatty acid biosynthesis)
rocB	3878	involved in arginine and ornithine utilization	ywhF	3849	spermidine synthase	accC	2531	acetyl-CoA carboxylase (biotin carboxyl carrier subunit) (long-chain fatty acid biosynthesis)
rocD	4145	ornithine aminotransferase (arginine and ornithine utilization)	ywhG	3848	agmatinase	accD	2531	acetyl-CoA carboxylase (biotin carboxylase subunit) (long-chain fatty acid biosynthesis)
rocF	4142	arginase (arginine and ornithine utilization)	ywrD	3720	γ -glutamyltransferase	acdA	3813	acyl-CoA dehydrogenase
serA	2410	phosphoglycerate dehydrogenase (serine biosynthesis)	yxeP	4057	aminoacylase	acpA	1665	acyl carrier protein (fatty acid biosynthesis)
serC	1076	phosphoserine aminotransferase (serine biosynthesis)	il3	METABOLISM OF NUCLEOTIDES AND NUCLEIC ACIDS	cdsA	1721	phosphatidate cytidyltransferase (phospholipid biosynthesis)	
tdh	1770	threonine 3-dehydrogenase (threonine catabolism)	adeC	1521	adenine deaminase	dgkA	2611	diacylglycerol kinase (phospholipid biosynthesis)
thrB	3313	homoserine kinase (threonine biosynthesis)	adeK	146	adenylate kinase	fabD	1663	malonyl CoA-acyl carrier protein transacylase (fatty acid biosynthesis)
thrC	3314	threonine synthase (threonine biosynthesis)	apt	2823	adenine phosphoribosyltransferase	fabG	1664	3-ketoacyl-acyl carrier protein reductase (fatty acid biosynthesis)
trpA	2372	tryptophan synthase (α subunit) (tryptophan biosynthesis)	cdd	2611	cytidine/deoxycytidine deaminase	glpQ	234	glycerophosphoryl diester phosphodiesterase (glycerol metabolism)
trpB	2373	tryptophan synthase (β subunit) (tryptophan biosynthesis)	cmk	2386	cytidylate kinase	lcfA	2919	long chain acyl-CoA synthetase (fatty acid metabolism)
trpC	2374	indol-3-glycerol phosphate synthase (tryptophan biosynthesis)	ctrA	2381	CTP synthetase (pyrimidine biosynthesis)	lipA	292	lipase
trpD	2375	anthranilate phosphoribosyltransferase (tryptophan biosynthesis)	deoD	2135	purine nucleoside phosphorylase (purine nucleoside salvage)	lipB	910	lipase
trpE	2377	anthranilate synthase (tryptophan biosynthesis)	dra	4051	deoxyribose-phosphate aldolase (nucleotide/deoxyribonucleotide catabolism)	mmgA	2513	acetyl-CoA acetyltransferase
trpF	2373	phosphoribosyl anthranilate isomerase (tryptophan biosynthesis)	drm	2448	phosphodeoxyribomutase (purine nucleoside salvage)	mmgB	2512	3-hydroxybutyryl-CoA dehydrogenase
tyrA	2370	phenylalanine dehydrogenase (tyrosine biosynthesis)	guaA	692	GMP synthetase (GMP biosynthesis)	mmgC	2511	acyl-CoA dehydrogenase
ureA	3768	urease (γ subunit)	guaB	16	inositol-monophosphate dehydrogenase (GMP biosynthesis)	nag	593	carboxylesterase NA
ureB	3768	urease (β subunit)	hipO	3000	hippurate hydrolase	pgsA	1762	phosphatidylglycerophosphate synthase (acidic phospholipid biosynthesis)
ureC	3767	urease (α subunit)	hprT	76	hypoxanthine-guanine phosphoribosyltransferase (purine salvage)	plsX	1662	involved in fatty acid/phospholipid synthesis
vpr	3907	major extracellular serine protease	htrT	76	hypoxanthine-guanine phosphoribosyltransferase (purine salvage)	pnbA	3530	p-nitrobenzyl esterase
yaaO	38	lysine decarboxylase	ndk	2381	nucleoside diphosphate kinase	psd	249	phosphatidylserine decarboxylase (phospholipid biosynthesis)
ybgE	259	branched-chain amino acid aminotransferase	nin	372	inhibitor of the DNA degrading activity of NucA	pssA	248	phosphatidylserine synthase (phospholipid biosynthesis)
ybgJ	265	glutaminase	nrdE	1868	ribonucleoside-diphosphate reductase (major subunit)	sqhC	2101	squalene-hopene cyclase (hopanoid metabolism)
yccC	290	asparaginase	nrdF	1870	ribonucleoside-diphosphate reductase (minor subunit)	ybkK	247	carboxylesterase
ycgM	344	proline oxidase	nucA	372	membrane-associated nuclease	yclB	412	phenylacrylic acid decarboxylase
ycgN	345	1-pyrroline-5-carboxylate dehydrogenase	nucB	2652	sporulation-specific extracellular nuclease	ydbM	505	butyryl-CoA dehydrogenase
yclE	415	prolyl aminopeptidase	nucC	2652	sporulation-specific extracellular nuclease	ydbB	515	holo-acyl-carrier protein synthase
yclM	432	homoserine dehydrogenase	pdp	4049	pyrimidine-nucleoside phosphorylase	yfrR	871	3-hydroxyisobutyrate dehydrogenase
ycnG	441	4-aminobutyrate aminotransferase	pnp	2446	purine nucleoside phosphorylase (purine nucleoside salvage)	yhaR	1061	3-hydroxybutyryl-CoA dehydratase
ycnH	443	succinate-semialdehyde dehydrogenase	pnpA	1739	polynucleotide phosphorylase	yhdO	1031	1-acylglycerol-3-phosphate O-acyltransferase
ycsA	452	3-isopropylmalate dehydrogenase	prs	58	phosphoribosyl pyrophosphate synthetase (nucleotide biosynthesis)	yhdW	1038	glycerophosphodiester phosphodiesterase
ycsL	459	allophanate hydrolase	purA	4156	adenylosuccinate synthetase (AMP biosynthesis)	yhbB	1093	3-oxoacyl-acyl-carrier protein synthase
yedD	718	glutamate synthase (ferredoxin)	purB	700	adenylosuccinate lyase (purine biosynthesis)	yhl	1099	lipate-protein ligase
yerM	729	amidase	purC	701	phosphoribosylaminoimidazole succinocarboxamide synthetase (purine biosynthesis)	yhlL	1100	long-chain fatty acid-CoA ligase
yhaA	1081	aminoacylase	purD	710	phosphoribosylglycinamide synthetase (purine biosynthesis)	yhlM	1110	acetyl-CoA C-acetyltransferase
yhdR	1034	aspartate aminotransferase	purE	698	phosphoribosylaminoimidazole carboxylase I (purine biosynthesis)	yhtT	1111	long-chain fatty acid-CoA ligase
yheM	1041	D-alanine aminotransferase	purF	705	phosphoribosylpyrophosphate amidotransferase (purine biosynthesis)	yisP	1159	phytoene synthase
yisK	1152	5-oxo-1,2,5-tricarboxylic-3-penten acid decarboxylase	purH	708	phosphoribosylaminoimidazole carboxy formyl transferase / inosine-monophosphate cyclohydrolase (purine biosynthesis)	yjaX	1208	3-oxoacyl-acyl-carrier protein synthase
yisO	1157	asparagine synthase	purI	699	phosphoribosylaminoimidazole carboxylase II (purine biosynthesis)	yjaY	1209	3-oxoacyl-acyl-carrier protein synthase
yisW	1167	opine aminotransferase	purL	702	phosphoribosylformylglycinamide synthetase II (purine biosynthesis)	yjbW	1247	enoyl-acyl-carrier protein reductase
yjbG	1231	oligoendopeptidase	purM	706	phosphoribosylaminoimidazole synthetase (purine biosynthesis)	yjdA	1268	3-oxoacyl-acyl-carrier protein reductase
yjbR	1243	sarcosine oxidase	purN	708	phosphoribosylglycinamide formyltransferase (purine biosynthesis)	ykhA	1372	acyl-CoA hydrolase
yjcL	1258	cystathionine γ -synthase	purQ	703	phosphoribosylglycinamide formyltransferase (purine biosynthesis)	ykwC	1465	3-hydroxyisobutyrate dehydrogenase
yjcl	1259	cystathionine β -lyase	purR	703	phosphoribosylformylglycinamide synthetase I (purine biosynthesis)	ymfI	1759	3-oxoacyl-acyl-carrier protein reductase
ykeA	1359	pyrroline-5-carboxylate reductase	purT	244	phosphoribosylglycinamide formyltransferase 2 (purine biosynthesis)	yngF	1951	3-hydroxybutyryl-CoA dehydratase
ykrV	1425	aspartate aminotransferase	pyrAA	1622	carbamoyl-phosphate synthetase (glutaminase subunit) (pyrimidine biosynthesis)	yngG	1952	hydroxybutyryl-CoA lyase
ykuQ	1488	tetrahydrodipicolinate succinylase	pyrAB	1623	carbamoyl-phosphate synthetase (catalytic subunit) (pyrimidine biosynthesis)	yngI	1955	long-chain acyl-CoA synthetase
ykuR	1489	hippurate hydrolase	pyrB	1620	aspartate carbamoyltransferase (pyrimidine biosynthesis)	yocE	2089	fatty acid desaturase
yldM	1551	glutaminase	pyrC	1621	dihydroorotate (pyrimidine biosynthesis)	yocJ	2096	ACP phosphodiesterase
ylnB	1607	acetylornithine deacetylase	pyrD	1627	dihydroorotate dehydrogenase (pyrimidine biosynthesis)	yodR	2143	butyrate-acetoacetate CoA-transferase
ylnW	1658	phosphoglycerate dehydrogenase	pyrDI	1626	dihydroorotate dehydrogenase (electron transfer subunit) (pyrimidine biosynthesis)	yodS	2144	3-oxoadipate CoA-transferase
ylnA	1658	L-serine dehydratase	pyrE	1629	orotate phosphoribosyltransferase (pyrimidine biosynthesis)	yoxD	2019	3-oxoacyl-acyl-carrier protein reductase
ymlG	1757	processing protease	pyrF	1628	orotidine 5'-phosphate decarboxylase (pyrimidine biosynthesis)	yqjD	2526	geranyltransferase
ymlH	1758	processing protease	relA	2822	GTP pyrophosphokinase (stringent response)	yqjK	2514	glycerophosphodiester phosphodiesterase
ymxG	1742	processing protease	smbA	1719	uridylic kinase (pyrimidine biosynthesis)	yqjS	2504	phosphate butyryltransferase
ynal	1885	phosphoribosylanthranilate isomerase	tdk	3802	thymidine kinase	yqjU	2502	branched-chain fatty acid kinase
yncD	1898	alanine racemase	thyA	1901	thymidylate synthase A (deoxyribonucleotide biosynthesis)	yqjQ	2471	ketoadyl reductase
yobN	2074	L-amino acid oxidase	thyB	2297	thymidylate synthase B (deoxyribonucleotide biosynthesis)	ysbB	2917	3-hydroxybutyryl-CoA dehydratase
yodT	2145	adenosylmethionine-8-amino-7-oxononanoate aminotransferase	tmk	39	thymidylate kinase	ytkK	3011	3-oxoacyl-acyl-carrier protein reductase
yopA	2403	glutamate dehydrogenase	udk	2792	uridine kinase (pyrimidine salvage)	ytpA	3123	lysophospholipase
yopW	2321	carboxypeptidase	upp	3788	uracil phosphoribosyltransferase (pyrimidine salvage)	yusJ	3368	butyryl-CoA dehydrogenase
yqel	2644	dihydrodipicolinate reductase				yusK	3369	acetyl-CoA C-acyltransferase
yqhl	2549	aminomethyltransferase				yusL	3372	3-hydroxyacyl-CoA dehydrogenase
yqhJ	2547	glycine dehydrogenase				yusQ	3376	acyloate catabolism
yqhK	2546	glycine dehydrogenase				yusR	3376	3-oxoacyl-acyl-carrier protein reductase
yqhS	2539	3-dehydroquininate dehydratase				yusS	3377	3-oxoacyl-acyl-carrier protein reductase
yqIT	2503	leucine dehydrogenase				yusG	3450	3-oxoacyl-acyl-carrier protein reductase
						yusD	3404	ketoadyl-carrier protein reductase
						ywhF	3866	3-oxoacyl-acyl-carrier protein reductase
						ywhB	3853	4-oxalocrotonate tautomerase
						ywiE	3822	cardiolipin synthetase
						ywiE	3816	cardiolipin synthetase
						ywnE	3762	cardiolipin synthase

<i>ywpB</i>	3743	hydroxymyristoyl-(acyl carrier protein) dehydrogenase	<i>yjbT</i>	1245	thiamin biosynthesis	<i>recR</i>	29	DNA repair and genetic recombination
<i>yxD</i>	4001	3-oxoadipate CoA-transferase	<i>yjbU</i>	1246	thiamin biosynthesis	<i>ruvA</i>	2636	Holliday junction DNA helicase
<i>yxE</i>	4001	3-oxoadipate CoA-transferase	<i>yjbV</i>	1246	thiamin biosynthesis	<i>ruvB</i>	2636	Holliday junction DNA helicase
			<i>ykpB</i>	1513	thiamin biosynthesis	<i>sbcD</i>	1143	endonuclease SbcD homologue
			<i>ykvK</i>	1440	6-pyruvoyl tetrahydrobiopterin synthase	<i>ylpB</i>	1659	ATP-dependent DNA helicase
II.5		METABOLISM OF COENZYMES AND PROSTHETIC GROUPS 99	<i>ykvL</i>	1440	coenzyme PQQ synthase	<i>yocI</i>	2095	ATP-dependent DNA helicase
<i>bioA</i>	3094	adenosylmethionine-8-amino-7-oxononanoate aminotransferase (biotin biosynthesis)	<i>ykbQ</i>	1577	pyrimidine-thiamine biosynthesis	<i>york</i>	2180	single-strand DNA-specific exonuclease
<i>bioB</i>	3091	biotin synthetase (biotin biosynthesis)	<i>ylnD</i>	1633	uroporphyrin-III C-methyltransferase	<i>yqhH</i>	2549	SNF2 helicase
<i>bioD</i>	3091	dethiobiotin synthetase (biotin biosynthesis)	<i>ylnF</i>	1635	uroporphyrin-III C-methyltransferase	<i>yrrC</i>	2808	conjugation transfer protein
<i>bioF</i>	3092	8-amino-7-oxononanoate synthase (biotin biosynthesis)	<i>yiol</i>	1642	pantothenate metabolism flavoprotein	<i>yrvE</i>	2825	single-strand DNA-specific exonuclease
<i>bioI</i>	3089	cytochrome P450-like enzyme (biotin biosynthesis)	<i>yngH</i>	1954	biotin carboxylase	<i>yvwA</i>	3735	SNF2 helicase
<i>bioW</i>	3094	6-carboxyhexanoate-CoA ligase (biotin biosynthesis)	<i>yodC</i>	2127	nitroreductase			
<i>dtfA</i>	2296	dihydrofolate reductase (glycine/purine/DNA precursor synthesis, conversion of dUMP to dTMP)	<i>yqgV</i>	2574	5-formyltetrahydrofolate cyclo-ligase	III.4		DNA PACKAGING AND SEGREGATION 10
<i>dhaS</i>	2100	aldehyde dehydrogenase	<i>yqS</i>	2469	pantothenate kinase	<i>grlA</i>	1935	DNA gyrase-like protein (subunit A)
<i>dhaB</i>	3291	2,3-dihydro-2,3-dihydroxybenzoate dehydrogenase (2,3-dihydroxybenzoate biosynthesis)	<i>yrrL</i>	2795	folate metabolism	<i>grlB</i>	1933	DNA gyrase-like protein (subunit B)
<i>dhaB</i>	3288	isochorismatase (2,3-dihydroxybenzoate biosynthesis)	<i>yrrM</i>	2795	caffeoyl-CoA O-methyltransferase	<i>gyrA</i>	7	DNA gyrase (subunit A)
<i>dhaC</i>	3291	isochorismatase synthase (2,3-dihydroxybenzoate biosynthesis)	<i>yueD</i>	3265	sepiapterin reductase	<i>gyrB</i>	5	DNA gyrase (subunit B)
<i>dhaE</i>	3289	2,3-dihydroxybenzoate-AMP ligase (enterobactin synthetase component E) (2,3-dihydroxybenzoate biosynthesis)	<i>yueI</i>	3261	pyrazinamidase/nicotinamidase	<i>hbs</i>	2385	non-specific DNA-binding protein HBSu
			<i>yueK</i>	3260	nicotinate phosphoribosyltransferase	<i>smc</i>	1666	chromosome segregation SMC protein homologue
<i>dhaF</i>	3287	involved in 2,3-dihydroxybenzoate biosynthesis	<i>yulG</i>	3293	biotin metabolism			
<i>folA</i>	87	7,8-dihydro-6-hydroxymethylpterin pyrophosphokinase (dihydrofolate biosynthesis)	<i>yurB</i>	3335	4-hydroxybenzoyl-CoA reductase	<i>smf</i>	1682	DNA processing Sml protein homologue
<i>folC</i>	2866	folyl-polyglutamate synthetase (folate biosynthesis)	<i>yurC</i>	3338	4-hydroxybenzoyl-CoA reductase	<i>topA</i>	1683	DNA topoisomerase I
<i>folD</i>	2529	methylene tetrahydrofolate dehydrogenase / methenyltetrahydrofolate cyclohydrolase (purines and amino acids biosynthesis)	<i>yurD</i>	3338	4-hydroxybenzoyl-CoA reductase	<i>topB</i>	476	DNA topoisomerase III
			<i>yutB</i>	3320	lipic acid synthetase	<i>yonN</i>	2225	HU-related DNA-binding protein
<i>folK</i>	87	7,8-dihydro-6-hydroxymethylpterin pyrophosphokinase (dihydrofolate biosynthesis)	<i>ywaB</i>	3950	quinone biosynthesis			
<i>ggt</i>	2004	γ -glutamyltranspeptidase (glutathione metabolism)	<i>ywkE</i>	3796	protoporphyrinogen oxidase			
			<i>ywoC</i>	3755	isochorismatase	III.5		RNA SYNTHESIS 244
<i>gsaB</i>	943	glutamate-1-semialdehyde aminotransferase	II.6		METABOLISM OF PHOSPHATE 9	III.5.1		INITIATION 19
<i>hemA</i>	2878	glutamyl-tRNA reductase (porphyrin biosynthesis)	<i>phoA</i>	1018	alkaline phosphatase A	<i>sigA</i>	2601	RNA polymerase major sigma factor (σ^70)
<i>hemB</i>	2874	8-aminolevulinic acid dehydratase (porphyrin biosynthesis)	<i>phoB</i>	621	alkaline phosphatase III	<i>sigB</i>	522	RNA polymerase general stress sigma factor (σ^32)
<i>hemC</i>	2876	porphobilinogen deaminase (porphyrin biosynthesis)	<i>phoD</i>	284	phosphodiesterase/alkaline phosphatase	<i>sigD</i>	1716	RNA polymerase flagella, motility, chemotaxis and autolysis sigma factor (σ^22)
<i>hemD</i>	2875	uroporphyrinogen III cosynthase (porphyrin biosynthesis)	<i>phoH</i>	2615	phosphate starvation-induced protein	<i>sigE</i>	1604	RNA polymerase sporulation mother cell-specific (early) sigma factor (σ^54) (SpoIIIG)
<i>hemE</i>	1086	uroporphyrinogen III decarboxylase (porphyrin biosynthesis)	<i>xpaC</i>	36	hydrolysis of 5-bromo-4-chloroindolyl phosphate	<i>sigF</i>	2443	RNA polymerase sporulation forespore-specific (early) sigma factor (σ^70) (SpoIIAC)
<i>hemH</i>	1087	ferrochelatase (porphyrin biosynthesis)	<i>ybfM</i>	248	alkaline phosphatase	<i>sigG</i>	1605	RNA polymerase sporulation forespore-specific (late) sigma factor (σ^70) (SpoIIIG)
<i>hemL</i>	2873	glutamate-1-semialdehyde 2,1-aminotransferase (porphyrin biosynthesis)	<i>ykoX</i>	1409	alkaline phosphatase	<i>sigH</i>	117	RNA polymerase vegetative and early stationary-phase sigma factor (σ^70) (SpoIIH)
<i>hemN</i>	2630	coproporphyrinogen III oxidase (porphyrin biosynthesis)	<i>yiaK</i>	1549	phosphate starvation inducible protein	<i>sigI</i>	3513	RNA polymerase sigma factor (σ^70)
<i>hemX</i>	2877	negative effector of the concentration of HemA	<i>yngC</i>	1947	alkaline phosphatase	<i>sigV</i>	2769	RNA polymerase ECF-type sigma factor (σ^54)
<i>hemY</i>	1088	protoporphyrinogen IX oxidase (porphyrin biosynthesis)	II.7		METABOLISM OF SULPHUR 3	<i>sigW</i>	195	RNA polymerase ECF-type sigma factor (σ^54)
<i>menB</i>	3149	dihydroxynaphthoic acid synthetase (menaquinone biosynthesis)	<i>yisZ</i>	1170	adenylsulfate kinase	<i>sigX</i>	2414	RNA polymerase ECF-type sigma factor (σ^54)
<i>menD</i>	3151	2-succinyl-6-hydroxy-2,4-cyclohexadiene-1-carboxylate synthase / 2-oxoglutarate decarboxylase (menaquinone biosynthesis)	<i>yitA</i>	1171	sulfate adenyltransferase	<i>sigY</i>	3970	RNA polymerase ECF-type sigma factor (σ^54)
<i>menE</i>	3148	O-succinylbenzoic acid-CoA ligase (menaquinone biosynthesis)	<i>yitB</i>	1172	phospho-adenylsulfate sulfotransferase	<i>sigZ</i>	2742	RNA polymerase ECF-type sigma factor (σ^54)
<i>menF</i>	3153	menaquinone-specific isochorismatase synthase (menaquinone biosynthesis)	<i>yitC</i>	1632	sulfate adenyltransferase	<i>spolIIC</i>	2701	RNA polymerase sporulation mother cell-specific (late) sigma factor (σ^70) (C-terminal half)
<i>moaB</i>	3014	molybdopterin precursor biosynthesis	<i>ylnC</i>	1633	adenylsulfate kinase	<i>spolIIC</i>	2652	RNA polymerase sporulation mother cell-specific (late) sigma factor (σ^70) (N-terminal half)
<i>moaD</i>	1499	molybdopterin converting factor (subunit 1)	<i>yulH</i>	3293	sulfite oxidase	<i>xpf</i>	1324	RNA polymerase PBX sigma factor-like
<i>moaE</i>	1498	molybdopterin converting factor (subunit 2)	<i>yvgQ</i>	3431	sulfite reductase	<i>yhdM</i>	1030	RNA polymerase ECF-type sigma factor
<i>moaF</i>	1495	molybdopterin-guanine dinucleotide biosynthesis	<i>yvgR</i>	3433	sulfite reductase	<i>ykoZ</i>	1411	RNA polymerase sigma factor
<i>moaB</i>	1498	molybdopterin-guanine dinucleotide biosynthesis	III		INFORMATION PATHWAYS 482	<i>yiaC</i>	1543	RNA polymerase ECF-type sigma factor
<i>moaF</i>	1497	molybdopterin biosynthesis protein	III.1		DNA REPLICATION 22			
<i>moaB</i>	1496	molybdopterin biosynthesis protein	<i>dnaA</i>	0	initiation of chromosome replication	III.5.2		REGULATION 213
<i>mtA</i>	2385	GTP cyclohydrolase I (tetrahydrofolate biosynthesis)	<i>dnaB</i>	2965	initiation of chromosome replication / membrane attachment protein	1517		transcriptional regulator of transition state genes (AbrB-like)
<i>nadA</i>	2846	nicotinate synthetase (quinolinate biosynthesis)	<i>dnaC</i>	4158	replicative DNA helicase	<i>abrB</i>	45	transcriptional pleiotropic regulator of transition state genes (<i>aprE</i> , <i>comK</i> , <i>ftsAZ</i> , <i>hpr</i> , <i>motAB</i> , <i>npfE</i> , <i>pbpE</i> , <i>rbs</i> , <i>spo0H</i> , <i>spoVG</i> , <i>spoVE</i> , <i>tycA</i>)
<i>nadB</i>	2849	nicotinate oxidase (quinolinate biosynthesis)	<i>dnaD</i>	2345	initiation of chromosome replication	<i>acoR</i>	883	transcriptional activator of the acetoin dehydrogenase operon (<i>acoABCD</i>)
<i>nadC</i>	2847	nicotinate-nucleotide pyrophosphorylase (NAD/NADP biosynthesis)	<i>dnaE</i>	2994	DNA polymerase III (α subunit)	<i>ahrC</i>	2522	transcriptional regulator of arginine metabolism expression (<i>roc</i> operon)
<i>nadE</i>	338	NH ₄ ⁺ -dependent NAD ⁺ synthetase (NAD biosynthesis)	<i>dnaG</i>	2603	DNA primase	<i>alsR</i>	3711	transcriptional regulator of the α -acetolactate operon (<i>alsSD</i>)
<i>narA</i>	3772	molybdopterin precursor biosynthesis	<i>dnaI</i>	2963	primosome component (helicase loader)	<i>ansR</i>	2456	transcriptional repressor of the <i>ansAB</i> operon (Xre family)
<i>narF</i>	355	uroporphyrin-III C-methyltransferase (porphyrin biosynthesis)	<i>dnaJ</i>	2	DNA polymerase III (β subunit)	<i>araR</i>	3485	transcriptional repressor of the arabinose operon (<i>araBADLMNPO</i>)
<i>nifS</i>	2849	required for NAD biosynthesis	<i>dnaK</i>	27	DNA polymerase III (γ and δ subunits)	<i>azlB</i>	2729	transcriptional repressor of the <i>azlBCD</i> operon
<i>pabA</i>	84	p-aminobenzoate synthase glutamine amidotransferase (subunit B) / anthranilate synthase (subunit II) (folate and tryptophan biosynthesis)	<i>hoB</i>	41	DNA polymerase III (δ subunit)	<i>birA</i>	2355	transcriptional repressor of the biotin operon (<i>bioWAFDBI</i>) / biotin acetyl-CoA-carboxylase synthetase
<i>pabB</i>	83	p-aminobenzoate synthase (subunit A) (folate biosynthesis)	<i>polA</i>	2975	DNA polymerase I	<i>bltR</i>	2716	transcriptional regulator of the <i>bltD</i> operon
<i>pabC</i>	85	aminodeoxychorismate lyase (folate biosynthesis)	<i>polC</i>	1727	DNA polymerase III (α subunit)	<i>bmrR</i>	2495	transcriptional activator of the <i>bmrUR</i> operon
<i>panB</i>	2354	ketopantoate hydroxymethyltransferase (pantothenate biosynthesis)	<i>priA</i>	1643	primosomal replication factor Y	<i>ccpA</i>	3044	transcriptional regulator involved in carbon catabolite control
<i>panC</i>	2353	pantothenate synthetase (pantothenate biosynthesis)	<i>rh</i>	1677	ribonuclease H	<i>cheB</i>	1711	two-component response regulator-like [CheA] / methyl-accepting chemotaxis proteins-glutamate methyltransferase
<i>panD</i>	2352	aspartate 1-decarboxylase (pantothenate biosynthesis)	<i>rtp</i>	2018	replication terminator protein	<i>cheY</i>	1703	two-component response regulator [CheA] involved in modulation of flagellar switch bias (chemotaxis)
<i>ribA</i>	2429	GTP cyclohydrolase II / 3,4-dihydroxy-2-butanone 4-phosphate synthase (riboflavin biosynthesis)	<i>ssb</i>	4199	single-strand DNA-binding protein	<i>citR</i>	1020	transcriptional repressor of the citrate synthase I gene (<i>citA</i>)
<i>ribB</i>	2429	riboflavin synthase (α subunit) (riboflavin biosynthesis)	<i>yerF</i>	719	ATP-dependent DNA helicase	<i>citT</i>	832	two-component response regulator [CitS]
<i>ribC</i>	1737	riboflavin kinase / FAD synthase (riboflavin biosynthesis)	<i>yerg</i>	721	DNA ligase	<i>codY</i>	1690	transcriptional pleiotropic repressor (expression of <i>srfA</i> , <i>comK</i> , <i>dpp</i> , <i>gabP</i> , <i>hut</i> , <i>ureAB</i>)
<i>ribG</i>	2431	riboflavin-specific deaminase (riboflavin biosynthesis)	<i>yqoV</i>	2192	DNA ligase	<i>comA</i>	3253	two-component response regulator [ComP] of late competence genes / surfactin production
<i>ribH</i>	2428	riboflavin synthase (β subunit) (riboflavin biosynthesis)	<i>yqoY</i>	2179	DNA polymerase III (α subunit)	<i>comK</i>	1117	competence transcription factor (CTF), final autoregulatory control switch prior to competence development
<i>ribT</i>	2427	reductase (riboflavin biosynthesis)	<i>yrcL</i>	2311	5'-3' exonuclease	<i>comQ</i>	3256	transcriptional regulator of late competence operon (<i>comG</i>) and surfactin expression (<i>srfA</i>)
<i>sul</i>	86	dihydropterolate synthase (dihydrofolate biosynthesis)	<i>yrcP</i>	3740	single-strand DNA-binding protein	<i>ctsR</i>	101	transcriptional repressor of class III stress genes (<i>clpC</i> , <i>clpP</i>)
<i>thiA</i>	955	synthesis of the pyrimidine moiety of thiamin (thiamin biosynthesis)	<i>yrcQ</i>	3740	single-strand DNA-binding protein	<i>degA</i>	1163	transcriptional activator involved in the degradation of glutamine phosphoribosylpyrophosphate amidotransferase
<i>thiC</i>	3930	thiamine-phosphate pyrophosphorylase (thiamin biosynthesis)	<i>yrcD</i>	1255	ATP-dependent DNA helicase	<i>degU</i>	3644	two-component response regulator [DegS] involved in degradative enzyme and competence regulation (<i>sacB</i> , <i>degQ</i> , <i>comK</i>)
<i>thiD</i>	3930	phosphomethylpyrimidine kinase (thiamin biosynthesis)	<i>yrcE</i>	1290	mutator MutT protein	<i>deoR</i>	4052	transcriptional repressor of the <i>draI</i> / <i>nupC</i> / <i>pdp</i> operon (deoxyribonucleoside)
<i>thiK</i>	3931	hydroxyethylthiazole kinase (thiamin biosynthesis)	<i>yrcF</i>	1290	mutator MutT protein	<i>fnr</i>	3831	transcriptional regulator of anaerobic genes (<i>narK</i> , <i>narGHI</i>)
<i>yaal</i>	26	isochorismatase	<i>yrcG</i>	1290	mutator MutT protein	<i>fruR</i>	1507	transcriptional repressor of the fructose operon (<i>fruRBA</i>)
<i>ydiA</i>	640	thiamin-monophosphate kinase	<i>yrcH</i>	1290	mutator MutT protein	<i>gerE</i>	2904	transcriptional regulator required for expression of late spore coat genes
<i>ydiG</i>	646	molybdopterin precursor biosynthesis	<i>yrcI</i>	1290	mutator MutT protein	<i>glcR</i>	3739	transcriptional repressor involved in the expression of the phosphotransferase system
<i>yhaV</i>	1058	coproporphyrinogen III oxidase	<i>yrcJ</i>	1290	mutator MutT protein	<i>glcT</i>	1456	transcriptional antiterminator essential for the expression of the <i>ptsGHI</i> operon
<i>yhcB</i>	979	flavodoxin	<i>yrcK</i>	1290	mutator MutT protein	<i>glrR</i>	1877	transcriptional repressor of the glutamine synthetase gene (<i>glrA</i>)
<i>yhfU</i>	1112	biotin biosynthesis	<i>yrcL</i>	1290	mutator MutT protein	<i>glpP</i>	1001	transcriptional antiterminator and control of mRNA stability of <i>glpD</i>
<i>yhxA</i>	1000	adenosylmethionine-8-amino-7-oxononanoate aminotransferase	<i>yrcM</i>	1290	mutator MutT protein	<i>glpC</i>	2014	transcriptional activator of the glutamate synthase operon (<i>gluAB</i>)
			<i>recF</i>	3	DNA repair and genetic recombination	<i>glrR</i>	2725	transcriptional repressor of the glutamate synthase operon (<i>gluAB</i>)
			<i>recN</i>	2522	DNA repair and genetic recombination	<i>gntR</i>	4113	transcriptional repressor of the gluconate operon (<i>gntRKPF</i>)
			<i>recQ</i>	2408	ATP-dependent DNA helicase			

<i>gutR</i>	667	transcriptional activator of the sorbitol dehydrogenase gene (<i>gutA</i>)	<i>ydeC</i>	562	transcriptional regulator (AraC/XylS family)	III.5.4	TERMINATION.....4
<i>hpr</i>	1073	transcriptional repressor of sporulation and extracellular proteases genes (<i>aprE</i> , <i>nprE</i> , <i>sin</i>)	<i>ydeE</i>	564	transcriptional regulator (AraC/XylS family)	<i>nusA</i>	1732 transcription termination
<i>hrcA</i>	2629	transcriptional repressor of class I heat-shock genes (<i>dnaK</i> , <i>groESL</i>)	<i>ydeF</i>	571	transcriptional regulator (GntR family) / amino-transferase (MocR-like)	<i>nusG</i>	118 transcription antitermination factor
<i>hutP</i>	4040	transcriptional activator of the histidine utilization operon (<i>hutPHUIGA</i>)	<i>ydeL</i>	574	transcriptional regulator (GntR family) / amino-transferase (MocR-like)	<i>yqhZ</i>	3904 transcriptional terminator Rho
<i>iolR</i>	4084	transcriptional repressor of the myo-inositol catabolism operon (<i>iolABCDGHIJ</i>)	<i>ydeS</i>	578	transcriptional regulator (TetR/AcrR family)	<i>rho</i>	2529 transcription termination
<i>kdgR</i>	2325	transcriptional repressor of the pectin utilization operon (<i>kdgRKA</i>)	<i>ydeT</i>	579	transcriptional regulator (ArsR family)		
<i>lacR</i>	3509	transcriptional repressor of the β -galactosidase gene (<i>lacA</i>)	<i>ydeD</i>	583	transcriptional regulator (GntR family) / amino-transferase (MocR-like)		
<i>levR</i>	2765	transcriptional activator of the levanase operon (<i>levDEFG</i>)	<i>ydfI</i>	589	two-component response regulator [YdfH]	III.6	RNA MODIFICATION.....19
<i>lexA</i>	1918	transcriptional repressor of the SOS regulon	<i>ydgG</i>	609	transcriptional regulator (MarR family)	<i>cspR</i>	970 rRNA methylase homolog
<i>licR</i>	3963	transcriptional regulator (antiterminator) of the lichenan operon (<i>licBCA</i>)	<i>ydgJ</i>	613	transcriptional regulator (MarR family)	<i>deaD</i>	4016 ATP-dependent RNA helicase
<i>licT</i>	4012	transcriptional antiterminator required for substrate-dependent induction and catabolite repression of <i>bglPH</i>	<i>ydhC</i>	616	transcriptional regulator (GntR family)	<i>miaA</i>	1866 tRNA isopentenylpyrophosphate transferase
<i>lmrA</i>	290	transcriptional repressor of the lincomycin operon (<i>lmrBA</i>)	<i>ydhO</i>	630	transcriptional regulator (GntR family)	<i>queA</i>	2834 Sadenosylmethionine tRNA ribosyltransferase (queuosine biosynthesis)
<i>lrpA</i>	551	transcriptional Lrp-like regulator (repression of <i>glyA</i> transcription and KinB-dependent sporulation)	<i>yerO</i>	732	transcriptional regulator (TetR/AcrR family)	<i>mcs</i>	1665 ribonuclease III
<i>lrpB</i>	552	transcriptional Lrp-like regulator (repression of <i>glyA</i> transcription and KinB-dependent sporulation)	<i>yesN</i>	760	two-component response regulator [YesM]	<i>mpaA</i>	4214 ribonuclease P (protein component)
<i>lrpC</i>	476	transcriptional regulator (Lrp/AsnC family)	<i>yesS</i>	765	transcriptional regulator (AraC/XylS family)	<i>rph</i>	2901 tRNA-guanine transglycosylase (queuosine biosynthesis)
<i>lytR</i>	3662	attenuator role for <i>lytABC</i> and <i>lytR</i> expression	<i>yetL</i>	790	transcriptional regulator (MarR family)	<i>tgt</i>	2833 tRNA methyltransferase
<i>lytT</i>	2956	two-component response regulator [LytS] involved in the rate of autolysis	<i>yezC</i>	711	transcriptional regulator (Lrp/AsnC family)	<i>truA</i>	153 pseudouridylyl synthase I
<i>msmR</i>	3096	transcriptional activator of multidrug-efflux transporter genes (<i>mrpA</i> and <i>mrpB</i>)	<i>yfiF</i>	898	transcriptional regulator (AraC/XylS family)	<i>truB</i>	1736 tRNA pseudouridine 5S synthase
<i>mta</i>	3764	transcriptional activator of multidrug-efflux transporter genes (<i>mrpA</i> and <i>mrpB</i>)	<i>yfiK</i>	905	two-component response regulator [YfiJ]	<i>ydbR</i>	511 ATP-dependent RNA helicase
<i>mtrB</i>	2384	tryptophan operon RNA-binding attenuation protein (TRAP)	<i>yfiV</i>	916	transcriptional regulator (MarR family)	<i>yefA</i>	737 RNA methyltransferase
<i>paiA</i>	3304	transcriptional repressor of sporulation, septation and degradative enzyme genes (<i>aprE</i> , <i>nprE</i> , <i>phoA</i> , <i>sacB</i>)	<i>yfmP</i>	912	transcriptional regulator (MerR family)	<i>yjiO</i>	873 RNA methyltransferase
<i>paiB</i>	3304	transcriptional repressor of sporulation and degradative enzyme genes	<i>ygaG</i>	944	transcriptional regulator (Fur family)	<i>yjmL</i>	816 RNA helicase
<i>phoP</i>	2978	two-component response regulator [PhoR] involved in phosphate regulation (<i>phoA</i> , <i>phoB</i> , <i>phoD</i> , <i>resABCD</i>)	<i>yhbI</i>	976	transcriptional regulator (MarR family)	<i>yjOM</i>	1647 RNA-binding Sun protein
<i>pkasA</i>	1781	transcriptional regulator of the polyketide synthase operon (<i>pkas</i>)	<i>yhcF</i>	981	transcriptional regulator (GntR family)	<i>yjQR</i>	2595 ATP-dependent RNA helicase
<i>purR</i>	54	transcriptional repressor of the purine operon (<i>purEBCDQFMNH</i>)	<i>yhcZ</i>	1009	two-component response regulator [YhcY]	<i>yjsgA</i>	2331 rRNA methylase
<i>pyrR</i>	1618	transcriptional attenuation of the pyrimidine operon (<i>pyrPBCADFE</i>) / uracil phosphoribosyltransferase activity (minor) [pyrimidine biosynthesis]	<i>yhdI</i>	1027	transcriptional regulator (GntR family) / amino-transferase (MocR-like)	<i>yjgl</i>	3225 polyribonucleotide nucleotidyltransferase
<i>rbfR</i>	3700	transcriptional repressor of the ribose operon (<i>rbfRKDACB</i>)	<i>yhdQ</i>	1033	transcriptional regulator (MerR family)	III.7	PROTEIN SYNTHESIS.....96
<i>resD</i>	2417	two-component response regulator [ResE] involved in aerobic and anaerobic respiration	<i>yhgD</i>	1089	transcriptional regulator (TetR/AcrR family)	III.7.1	RIBOSOMAL PROTEINS.....56
<i>ribR</i>	3001	transcriptional regulator of riboflavin biosynthesis genes	<i>yhiM</i>	1129	transcriptional regulator (LacI family)	<i>rplA</i>	119 ribosomal protein L1 (BL1)
<i>rocR</i>	4145	transcriptional activator of arginine utilization operons (<i>rocABC</i> , <i>rocDEF</i>)	<i>yisR</i>	1162	transcriptional regulator (AraC/XylS family)	<i>rplB</i>	137 ribosomal protein L2 (BL2)
<i>sacT</i>	3906	transcriptional antiterminator involved in positive regulation of <i>sacA</i> and <i>sacP</i>	<i>yisV</i>	1166	transcriptional regulator (GntR family) / amino-transferase (MocR-like)	<i>rplC</i>	136 ribosomal protein L3 (BL3)
<i>sacV</i>	532	transcriptional regulator of the levansucrase gene (<i>sacB</i>)	<i>yjdC</i>	1270	transcriptional antiterminator (BglG family)	<i>rplD</i>	136 ribosomal protein L4
<i>sacY</i>	3942	transcriptional antiterminator involved in positive regulation of levansucrase and sucrose synthesis	<i>yjdI</i>	1277	transcription regulation	<i>rplE</i>	141 ribosomal protein L5 (BL6)
<i>senS</i>	959	transcriptional regulator of extracellular enzyme genes (<i>amyE</i> , <i>aprE</i> , <i>nprE</i>)	<i>yjhH</i>	1308	transcriptional regulator (LacI family)	<i>rplF</i>	142 ribosomal protein L6 (BL8)
<i>sinR</i>	2552	transcriptional regulator of post-exponential-phase responses genes (<i>aprE</i> , <i>comK</i> , <i>kinB</i> , <i>sigD</i> , <i>spo0A</i> , <i>spoIIA</i> , <i>spoIIE</i> , <i>spoIIG</i>)	<i>ykoG</i>	1391	two-component response regulator [YkoH]	<i>rplJ</i>	4163 ribosomal protein L9
<i>slr</i>	3529	transcriptional activator of competence development and sporulation genes	<i>ykoM</i>	1485	transcriptional regulator (MarR family)	<i>rplK</i>	120 ribosomal protein L10 (BL5)
<i>spIA</i>	1461	transcriptional regulator of the spore photoproduct lyase operon (<i>spIAB</i>)	<i>ykvE</i>	1433	transcriptional regulator (MarR family)	<i>rplL</i>	119 ribosomal protein L11 (BL11)
<i>spo0A</i>	2518	two-component response regulator [KinC] central for the initiation of sporulation (<i>spo0A</i> , <i>abrB</i> , <i>kinA</i> , <i>kinC</i> , <i>spoIIA</i> , <i>spoIIE</i> , <i>spoIIG</i>) (part of phosphorelay: Spo0B-P $\rightarrow$ Spo0A-P)	<i>ykvZ</i>	1455	transcriptional regulator (LacI family)	<i>rplM</i>	121 ribosomal protein L12 (BL9)
<i>spo0F</i>	3809	two-component response regulator [KinA, KinB] involved in the initiation of sporulation (part of phosphorelay: Spo0F-P $\rightarrow$ Spo0B-P)	<i>ymlC</i>	1754	transcriptional regulator (GntR family)	<i>rplN</i>	140 ribosomal protein L14
<i>spoIID</i>	3748	transcriptional regulator of σ^E - and σ^D -dependent genes	<i>yneI</i>	1923	two-component response regulator (CheY homologue)	<i>rplO</i>	144 ribosomal protein L15
<i>spoVT</i>	64	transcriptional positive and negative regulator of σ^E -dependent genes	<i>yocU</i>	2045	transcriptional regulator (LysR family)	<i>rplP</i>	139 ribosomal protein L16
<i>tenA</i>	1242	transcriptional activator of extracellular enzyme genes (<i>aprE</i> , <i>nprE</i> , <i>phoA</i> , <i>sacB</i>)	<i>yobD</i>	2056	transcriptional regulator (phage-related) (Xre family)	<i>rplQ</i>	150 ribosomal protein L17 (BL15)
<i>tenI</i>	1243	transcriptional activator of extracellular enzyme genes	<i>yobG</i>	2080	transcriptional regulator (AraC/XylS family)	<i>rplR</i>	143 ribosomal protein L18
<i>tnrA</i>	1397	transcriptional pleiotropic regulator involved in global nitrogen regulation (expression of <i>nrgAB</i> , <i>nasB</i> , <i>gabP</i> , <i>ureABC</i> , <i>glnRA</i>)	<i>yocG</i>	2091	two-component response regulator [YocF]	<i>rplS</i>	1675 ribosomal protein L19
<i>treR</i>	853	transcriptional repressor of the trehalose operon (<i>trePAR</i>)	<i>yocR</i>	2097	transcriptional regulator (LysR family)	<i>rplT</i>	2352 ribosomal protein L20
<i>xre</i>	1321	transcriptional repressor of PBX genes	<i>yocR</i>	2221	transcriptional regulator (phage-related) (Xre family)	<i>rplU</i>	2855 ribosomal protein L21 (BL20)
<i>xylR</i>	1891	transcriptional repressor of the xyllose operon (<i>xylAB</i>)	<i>yozA</i>	2084	transcriptional regulator (AraC/XylS family)	<i>rplV</i>	138 ribosomal protein L22 (BL17)
<i>yacF</i>	88	transcriptional regulator (nitrogen regulation protein)	<i>yozG</i>	2043	transcriptional regulator (AraC/XylS family)	<i>rplW</i>	137 ribosomal protein L23
<i>ybbB</i>	185	transcriptional regulator (AraC/XylS family)	<i>yozP</i>	2294	transcriptional regulator (σ^E -dependent)	<i>rplX</i>	141 ribosomal protein L24 (BL23) (histone-like protein HPB12)
<i>ybdI</i>	221	two-component response regulator [YbdK]	<i>yypP</i>	2281	transcriptional regulator (LysR family)	<i>rpmA</i>	2854 ribosomal protein L27 (BL24)
<i>ybfI</i>	244	transcriptional regulator (AraC/XylS family)	<i>yypO</i>	2287	transcriptional regulator (PilB family)	<i>rpmB</i>	1655 ribosomal protein L28
<i>ybpP</i>	251	transcriptional regulator (AraC/XylS family)	<i>yypU</i>	2414	negative regulator of σ^E activity	<i>rpmC</i>	140 ribosomal protein L29
<i>ybgA</i>	258	transcriptional regulator (GntR family)	<i>yqaE</i>	2698	transcriptional regulator (phage-related) (Xre family)	<i>rpmD</i>	144 ribosomal protein L30 (BL27)
<i>ycbB</i>	267	two-component response regulator [YcbA]	<i>yqjC</i>	2657	transcriptional regulator (AraC/XylS family)	<i>rpmE</i>	3802 ribosomal protein L31
<i>ycbG</i>	273	transcriptional regulator (GntR family)	<i>yqjN</i>	2591	transcriptional regulator (Fur family)	<i>rpmF</i>	1575 ribosomal protein L32
<i>ycbL</i>	278	two-component response regulator [YcbM]	<i>yqhN</i>	2543	transcriptional regulator (σ^E -dependent)	<i>rpmG</i>	117 ribosomal protein L33
<i>yccH</i>	296	two-component response regulator [YccG]	<i>yqjR</i>	2506	transcriptional regulator (σ^E -dependent)	<i>rpmH</i>	4215 ribosomal protein L34
<i>yccK</i>	320	transcriptional regulator (AraC/XylS family)	<i>yqkL</i>	2450	transcriptional regulator (Fur family)	<i>rpmI</i>	2952 ribosomal protein L35
<i>yccG</i>	341	transcriptional regulator (LysR family)	<i>yraB</i>	2755	transcriptional regulator (MerR family)	<i>rpmJ</i>	148 ribosomal protein L36 (ribosomal protein B)
<i>ycaA</i>	412	transcriptional regulator (LysR family)	<i>yraC</i>	2746	transcriptional regulator (LysR family)	<i>rpmK</i>	1717 ribosomal protein S2
<i>ycaI</i>	426	two-component response regulator [YcaI]	<i>yriH</i>	2777	transcriptional regulator (TetR/AcrR family)	<i>rpsC</i>	139 ribosomal protein S3 (BS3)
<i>ycnC</i>	438	transcriptional regulator (TetR/AcrR family)	<i>yriM</i>	2770	anti-sigma factor [σ^E]	<i>rpsD</i>	3035 ribosomal protein S4 (BS4)
<i>ycnF</i>	441	transcriptional regulator (GntR family) / amino-transferase (MocR-like)	<i>yryK</i>	2704	two-component response regulator [YrkQ]	<i>rpsE</i>	143 ribosomal protein S5
<i>ycnK</i>	449	transcriptional regulator (DeoR family)	<i>yryP</i>	2704	two-component response regulator [YrkQ]	<i>rpsF</i>	4199 ribosomal protein S6 (BS9)
<i>ycsO</i>	461	transcriptional regulator (LysR family)	<i>yryQ</i>	2704	two-component response regulator [YrkQ]	<i>rpsG</i>	130 ribosomal protein S7 (BS7)
<i>ycsD</i>	406	transcriptional regulator (GntR family) / amino-transferase (MocR-like)	<i>yryR</i>	2704	two-component response regulator [YrkQ]	<i>rpsH</i>	142 ribosomal protein S8 (BS8)
<i>ycaZ</i>	439	transcriptional antiterminator (BglG family)	<i>yryS</i>	2704	two-component response regulator [YrkQ]	<i>rpsI</i>	154 ribosomal protein S9
<i>ydcG</i>	499	two-component response regulator [YdcF]	<i>yryT</i>	2704	two-component response regulator [YrkQ]	<i>rpsJ</i>	135 ribosomal protein S10 (BS13)
<i>ydcN</i>	531	transcriptional regulator (phage-related) (Xre family)	<i>yryU</i>	2704	two-component response regulator [YrkQ]	<i>rpsK</i>	148 ribosomal protein S11 (BS11)
			<i>yryV</i>	2704	two-component response regulator [YrkQ]	<i>rpsL</i>	130 ribosomal protein S12 (BS12)
			<i>yryW</i>	2704	two-component response regulator [YrkQ]	<i>rpsM</i>	148 ribosomal protein S13
			<i>yryX</i>	2704	two-component response regulator [YrkQ]	<i>rpsN</i>	142 ribosomal protein S14
			<i>yryY</i>	2704	two-component response regulator [YrkQ]	<i>rpsO</i>	1738 ribosomal protein S15 (BS18)
			<i>yryZ</i>	2704	two-component response regulator [YrkQ]	<i>rpsP</i>	1673 ribosomal protein S16 (BS17)
			<i>yryA</i>	2704	two-component response regulator [YrkQ]	<i>rpsQ</i>	140 ribosomal protein S17 (BS16)
			<i>yryB</i>	2704	two-component response regulator [YrkQ]	<i>rpsR</i>	4198 ribosomal protein S18
			<i>yryC</i>	2704	two-component response regulator [YrkQ]	<i>rpsS</i>	138 ribosomal protein S19 (BS19)
			<i>yryD</i>	2704	two-component response regulator [YrkQ]	<i>rpsT</i>	2635 ribosomal protein S20 (BS20)
			<i>yryE</i>	2704	two-component response regulator [YrkQ]	<i>rpsU</i>	2620 ribosomal protein S21
			<i>yryF</i>	2704	two-component response regulator [YrkQ]	<i>ybfX</i>	129 ribosomal protein L7AE family
			<i>yryG</i>	2704	two-component response regulator [YrkQ]	<i>yhzA</i>	965 ribosomal protein L7AE family
			<i>yryH</i>	2704	two-component response regulator [YrkQ]	<i>ylyD</i>	1733 ribosomal protein L7AE family
			<i>yryI</i>	2704	two-component response regulator [YrkQ]		3631 ribosomal protein S30AE family
			<i>yryJ</i>	2704	two-component response regulator [YrkQ]	III.7.2	AMINOACYL-TRNA SYNTHETASES.....25
			<i>yryK</i>	2704	two-component response regulator [YrkQ]	<i>alaS</i>	2800 alanyl-tRNA synthetase
			<i>yryL</i>	2704	two-component response regulator [YrkQ]	<i>argS</i>	3834 arginyl-tRNA synthetase
			<i>yryM</i>	2704	two-component response regulator [YrkQ]	<i>asnS</i>	2347 asparaginyl-tRNA synthetase
			<i>yryN</i>	2704	two-component response regulator [YrkQ]	<i>aspS</i>	2815 aspartyl-tRNA synthetase
			<i>yryO</i>	2704	two-component response regulator [YrkQ]	<i>cysS</i>	113 cysteinyl-tRNA synthetase
			<i>yryP</i>	2704	two-component response regulator [YrkQ]	<i>glxX</i>	111 glutamyl-tRNA synthetase
			<i>yryQ</i>	2704	two-component response regulator [YrkQ]	<i>glyQ</i>	2608 glycyl-tRNA synthetase (α subunit)
			<i>yryR</i>	2704	two-component response regulator [YrkQ]	<i>glyS</i>	2607 glycyl-tRNA synthetase (β subunit)
			<i>yryS</i>	2704	two-component response regulator [YrkQ]	<i>hisS</i>	2817 histidyl-tRNA synthetase
			<i>yryT</i>	2704	two-component response regulator [YrkQ]	<i>hisZ</i>	3588 histidyl-tRNA synthetase
			<i>yryU</i>	2704	two-component response regulator [YrkQ]	<i>ileS</i>	1613 isoleucyl-tRNA synthetase
			<i>yryV</i>	2704	two-component response regulator [YrkQ]	<i>leuS</i>	3104 leucyl-tRNA synthetase
			<i>yryW</i>	2704	two-component response regulator [YrkQ]	<i>lysS</i>	89 lysyl-tRNA synthetase
			<i>yryX</i>	2704	two-component response regulator [YrkQ]	<i>metS</i>	46 methionyl-tRNA synthetase
			<i>yryY</i>	2704	two-component response regulator [YrkQ]	<i>pheS</i>	2330 phenylalanyl-tRNA synthetase (α subunit)
			<i>yryZ</i>	2704	two-component response regulator [YrkQ]	<i>pheT</i>	2929 phenylalanyl-tRNA synthetase (β subunit)
			<i>yryA</i>	2704	two-component response regulator [YrkQ]	<i>proS</i>	1725 prolyl-tRNA synthetase
			<i>yryB</i>	2704	two-component response regulator [YrkQ]	<i>serS</i>	21 seryl-tRNA synthetase
			<i>yryC</i>	2704	two-component response regulator [YrkQ]	<i>thrS</i>	2960 threonyl-tRNA synthetase (major)
			<i>yryD</i>	2704	two-component response regulator [YrkQ]	<i>thrZ</i>	3855 threonyl-tRNA synthetase (minor)
			<i>yryE</i>	2704	two-component response regulator [YrkQ]	<i>trpS</i>	1219 tryptophanyl-tRNA synthetase
			<i>yryF</i>	2704	two-component response regulator [YrkQ]	<i>tyrS</i>	3037 tyrosyl-tRNA synthetase (major)
			<i>yryG</i>	2704	two-component response regulator [YrkQ]	<i>tyrZ</i>	3946 tyrosyl-tRNA synthetase (minor)
			<i>yryH</i>	2704	two-component response regulator [YrkQ]	<i>valS</i>	2869 valyl-tRNA synthetase
			<i>yryI</i>	2704	two-component response regulator [YrkQ]	<i>yprP</i>	3052 phenylalanyl-tRNA synthetase (β subunit)
			<i>yryJ</i>	2704	two-component response regulator [YrkQ]	III.7.3	INITIATION.....6
			<i>yryK</i>	2704	two-component response regulator [YrkQ]	<i>fmt</i>	1646 methionyl-tRNA formyltransferase
			<i>yryL</i>	2704	two-component response regulator [YrkQ]	<i>infA</i>	148 initiation factor IF-1
			<i>yryM</i>	2704	two-component response regulator [YrkQ]	<i>infB</i>	1733 initiation factor IF-2
			<i>yryN</i>	2704	two-component response regulator [YrkQ]	<i>infC</i>	2952 initiation factor IF-3
			<i>yryO</i>	2704	two-component response regulator [YrkQ]	<i>rbfA</i>	1736 ribosome-binding factor A
			<i>yryP</i>	2704	two-component response regulator [YrkQ]	<i>ykrS</i>	1423 initiation factor eIF-2B (α subunit)
			<i>yryQ</i>	2704	two-component response regulator [YrkQ]	III.7.4	ELONGATION.....6
			<i>yryR</i>	2704	two-component response regulator [YrkQ]	<i>efp</i>	2538 elongation factor P
			<i>yryS</i>	2704	two-component response regulator [YrkQ]	<i>fus</i>	131 elongation factor G
			<i>yryT</i>	2704	two-component response regulator [YrkQ]		
			<i>yryU</i>	2704	two-component response regulator [YrkQ]		
			<i>yryV</i>	2704	two-component response regulator [YrkQ]		
			<i>yryW</i>	2704	two-component response regulator [YrkQ]		
			<i>yryX</i>	2704	two-component response regulator [YrkQ]		
			<i>yryY</i>	2704	two-component response regulator [YrkQ]		
			<i>yryZ</i>	2704	two-component response regulator [YrkQ]		
			<i>yryA</i>	2704	two-component response regulator [YrkQ]		
			<i>yryB</i>	2704	two-component response regulator [YrkQ]		

<i>lepA</i>	2632	GTP-binding protein	<i>yvE</i>	3515	spore coat polysaccharide biosynthesis	<i>xxdC</i>	1322	PBSX prophage
<i>tsf</i>	1718	elongation factor Ts	<i>yvB</i>	3384	serine protease Do	<i>xxdD</i>	1323	PBSX prophage
<i>tufA</i>	1531	elongation factor Tu	<i>ywqC</i>	3732	capsular polysaccharide biosynthesis	<i>xxdE</i>	1327	PBSX prophage
<i>yag</i>	1546	GTP-binding elongation factor	<i>ywqD</i>	3732	capsular polysaccharide biosynthesis	<i>xxdF</i>	1328	PBSX prophage
			<i>ywqE</i>	3731	capsular polysaccharide biosynthesis	<i>xxdG</i>	1329	PBSX prophage
III.7.5	TERMINATION	3	<i>ywsC</i>	3700	capsular polyglutamate biosynthesis	<i>xxdH</i>	1330	PBSX prophage
<i>frr</i>	1720	ribosome recycling factor	<i>ywtA</i>	3698	capsular polyglutamate biosynthesis	<i>xxdI</i>	1331	PBSX prophage
<i>prfA</i>	3797	peptide chain release factor 1	<i>ywtB</i>	3698	capsular polyglutamate biosynthesis	<i>xxdJ</i>	1331	PBSX prophage
<i>prfB</i>	3627	peptide chain release factor 2	<i>yyxA</i>	4148	serine protease Do	<i>xxdK</i>	1332	PBSX prophage
						<i>xxdM</i>	1333	PBSX prophage
III.8	PROTEIN MODIFICATION	27	IV.2	DETOXIFICATION	68	<i>xxdN</i>	1334	PBSX prophage
<i>amhX</i>	325	amidohydrolase	<i>aadK</i>	2736	aminoglycoside 6-adenyltransferase	<i>xxdO</i>	1334	PBSX prophage
<i>lgt</i>	3593	prolipoprotein diacylglycerol transferase (lipoprotein biosynthesis)	<i>ahpC</i>	4118	alkyl hydroperoxide reductase (small subunit)	<i>xxdP</i>	1338	PBSX prophage
			<i>ahpF</i>	4119	alkyl hydroperoxide reductase (large subunit)	<i>xxdQ</i>	1339	PBSX prophage
<i>map</i>	147	methionine aminopeptidase	<i>bmrU</i>	2493	NADH dehydrogenase	<i>xxdR</i>	1340	PBSX prophage
<i>pcp</i>	287	pyrrolidone-carboxylate peptidase				<i>xxdS</i>	1340	PBSX prophage
<i>ppbB</i>	2435	peptidyl-prolyl isomerase				<i>xxdT</i>	1341	PBSX prophage
<i>prkA</i>	973	serine protein kinase	<i>cah</i>	342	cephalosporin C deacetylase	<i>xxdU</i>	1342	PBSX prophage
<i>tgl</i>	3212	transglutaminase	<i>cypA</i>	2732	cytochrome P450-like enzyme	<i>xxdV</i>	1343	PBSX prophage
<i>ybdM</i>	224	protein kinase	<i>cypX</i>	3603	cytochrome P450-like enzyme	<i>xxdW</i>	1345	PBSX prophage
<i>ydiC</i>	642	glycoprotein endopeptidase	<i>katA</i>	960	vegetative catalase 1	<i>xxdX</i>	1345	PBSX prophage
<i>ydiD</i>	643	ribosomal-protein-alanine N-acetyltransferase	<i>katB</i>	4009	catalase 2	<i>xxdY</i>	1345	PBSX prophage lytic exoenzyme
<i>ydiE</i>	643	glycoprotein endopeptidase	<i>katX</i>	3964	catalase	<i>xtmA</i>	1325	PBSX terminase (small subunit)
<i>yfkI</i>	862	protein-tyrosine phosphatase	<i>ksgA</i>	51	dimethyladenosine transferase (kasugamycin resistance)	<i>xtmB</i>	1325	PBSX terminase (large subunit)
<i>yfIG</i>	840	methionine aminopeptidase				<i>xtxA</i>	1324	PBSX prophage
<i>yfjK</i>	1261	ribosomal-protein-alanine N-acetyltransferase	<i>mmr</i>	3657	methyleneomycin A resistance protein	<i>ycdD</i>	304	L-alanyl-D-glutamate peptidase
<i>ykbB</i>	1528	formylmethionine deformylase	<i>padC</i>	3532	ferulate decarboxylase	<i>ydcL</i>	530	integrase
<i>ykyY</i>	1453	Xaa-Pro dipeptidase	<i>penP</i>	2048	β -lactamase	<i>ydcM</i>	531	immunity region protein in prophage
<i>yloP</i>	1651	protein kinase	<i>pksS</i>	1659	hydrolyase of the polyketide produced by the pks cluster	<i>yhgE</i>	1090	phage infection protein
<i>yppP</i>	2287	peptide methionine sulfoxide reductase	<i>sodA</i>	2585	superoxide dismutase	<i>yjbl</i>	1235	lytic transglycosylase
<i>yqeT</i>	2624	ribosomal protein L11 methyltransferase	<i>sodF</i>	2103	superoxide dismutase	<i>yjgB</i>	1318	phage-related replication protein
<i>yqhT</i>	2539	Xaa-Pro dipeptidase	<i>tetL</i>	4188	tetracycline resistance leader peptide	<i>ymaC</i>	1863	phage-related protein
<i>ytel</i>	3020	protease IV	<i>thdF</i>	4212	thiophen and furan oxidation	<i>ymaH</i>	1867	host factor-1 protein
<i>ytiP</i>	3068	Xaa-His dipeptidase	<i>tmrB</i>	339	unicarmycin resistance	<i>ymlD</i>	1755	phage-related protein
<i>yvtA</i>	3105	protein kinase	<i>yaaN</i>	36	toxic cation resistance	<i>ymlE</i>	1756	phage-related protein
<i>yxtM</i>	3150	prolyl aminopeptidase	<i>ydeE</i>	190	β -lactamase	<i>ymlL</i>	1914	phage-related replication protein
<i>yuiE</i>	3297	leucyl aminopeptidase	<i>ybfO</i>	205	erythromycin esterase	<i>yobO</i>	2075	phage-related pre-neck appendage protein
<i>yviE</i>	3791	protein-tyrosine phosphatase	<i>yblO</i>	229	β -lactamase	<i>yokA</i>	2284	DNA recombinase
<i>yyaL</i>	4102	serine/threonine protein kinase	<i>yblI</i>	276	viomycin phosphotransferase	<i>yokL</i>	2274	phage-related protein
			<i>ycbR</i>	283	toxic cation resistance protein	<i>yolB</i>	2272	phage-related protein
III.9	PROTEIN FOLDING	8	<i>yceC</i>	312	tellurium resistance protein	<i>yomA</i>	2264	holin
<i>dnaK</i>	2627	class I heat-shock protein (chaperonin)	<i>yceD</i>	312	tellurium resistance protein	<i>yomJ</i>	2248	phage-related immunity protein
<i>groEL</i>	650	class I heat-shock protein (chaperonin)	<i>yceE</i>	313	tellurium resistance protein	<i>yomP</i>	2243	phage-related protein
<i>groES</i>	650	class I heat-shock protein (chaperonin)	<i>yceF</i>	314	tellurium resistance protein	<i>yomR</i>	2242	phage-related protein
<i>tig</i>	2887	trigger factor (prolyl isomerase)	<i>yceH</i>	316	toxic anion resistance protein	<i>yomS</i>	2241	phage-related lytic exoenzyme
<i>ykkC</i>	1376	chaperonin	<i>yceI</i>	467	lactam utilization protein	<i>yqqD</i>	2200	phage-related DNA-binding protein anti-repressor
<i>ykkD</i>	1376	chaperonin	<i>yddB</i>	496	manganese-containing catalase	<i>yqzQ</i>	2190	phage-related protein
<i>ykdR</i>	3541	chaperonin	<i>ydiB</i>	581	antibiotic resistance protein	<i>yqzS</i>	2160	phage-related endonuclease
<i>yvdS</i>	3541	chaperonin	<i>ydiE</i>	618	macrolide glycosyltransferase	<i>yqzB</i>	2700	phage-related protein
IV	OTHER FUNCTIONS	289	<i>yerP</i>	732	acriflavine resistance protein	<i>yqal</i>	2696	phage-related protein
IV.1	ADAPTATION TO ATYPICAL CONDITIONS	72	<i>yetM</i>	790	salicylate 1-monooxygenase	<i>yqak</i>	2695	phage-related protein
<i>bssA</i>	2304	glutathione peroxidase	<i>yetO</i>	792	cytochrome P450 / NADPH-cytochrome P450 reductase	<i>yqam</i>	2694	phage-related protein
<i>clpC</i>	104	class III stress response-related ATPase (repressor of competence)	<i>yilM</i>	836	nitric-oxide synthase	<i>yqaO</i>	2692	phage-related protein
<i>clpE</i>	1437	ATP-dependent Clp protease-like	<i>yinC</i>	804	fosmidmycin resistance protein	<i>yqaS</i>	2690	phage-related terminase large subunit
<i>clpP</i>	3545	ATP-dependent Clp protease proteolytic subunit (class III heat-shock protein)	<i>ygaF</i>	943	thiol-specific antioxidant protein	<i>yqaT</i>	2689	phage-related terminase small subunit
<i>clpQ</i>	1688	B-type subunit of the 20S proteasome	<i>yhiG</i>	1122	monooxygenase	<i>yqbA</i>	2688	phage-related protein
<i>clpX</i>	2885	ATP-dependent Clp protease ATP-binding subunit (class III heat-shock protein)	<i>yisY</i>	1169	chloride peroxidase	<i>yqbD</i>	2684	phage-related protein
<i>clpY</i>	1688	ATP-dependent Clp protease-like	<i>yjiB</i>	1291	monooxygenase	<i>yqde</i>	2683	phage-related protein
<i>csbB</i>	930	stress response protein	<i>yjiG</i>	1292	macrolide glycosyltransferase	<i>yqdeH</i>	2682	phage-related protein
<i>csbP</i>	984	major cold-shock protein	<i>ykfA</i>	1366	immunity to bacteriotoxins	<i>yqbl</i>	2681	phage-related protein
<i>csbC</i>	559	cold-shock protein	<i>ykkB</i>	1375	N-acetyltransferase	<i>yqbl</i>	2681	phage-related protein
<i>csbD</i>	2307	cold-shock protein	<i>ykoY</i>	1410	toxic anion resistance protein	<i>yqkK</i>	2680	phage-related protein
<i>cstA</i>	2937	carbon starvation-induced protein	<i>yndN</i>	1916	fosfomycin resistance protein	<i>yqbl</i>	2679	phage-related protein
<i>ctc</i>	59	general stress protein	<i>yocD</i>	2088	immunity to bacteriotoxins	<i>yqbN</i>	2679	phage-related protein
<i>degQ</i>	3256	degradative enzyme production	<i>yoiK</i>	2117	macrolide glycosyltransferase	<i>yqbO</i>	2677	phage-related protein
<i>degR</i>	3268	degradative enzyme production	<i>yoiM</i>	2115	superoxide dismutase	<i>yqbP</i>	2672	phage-related protein
<i>dnaI</i>	2625	heat-shock protein (activation of DnaK)	<i>yokD</i>	2281	aminoglycoside N ³ -acetyltransferase	<i>yqbQ</i>	2671	phage-related protein
<i>dsb</i>	3136	stress- and starvation-induced gene controlled by	<i>yqcm</i>	2655	arsenate reductase	<i>yqBR</i>	2670	phage-related protein
			<i>yqpP</i>	2596	penicillin tolerance	<i>yqbs</i>	2670	phage-related protein
			<i>yrfJ</i>	2776	cytochrome P450 / NADPH-cytochrome P450 reductase	<i>yqbt</i>	2670	phage-related protein
<i>gbsA</i>	3186	glycine betaine aldehyde dehydrogenase (osmoprotection)	<i>ytpB</i>	2736	2-nitropropane dioxygenase	<i>yqcA</i>	2669	phage-related protein
<i>gbsB</i>	3184	alcohol dehydrogenase (osmoprotection)	<i>ytlI</i>	3017	thiol peroxidase	<i>yqcC</i>	2668	phage-related protein
<i>gtpE</i>	2628	heat-shock protein (activation of DnaK)	<i>ytnI</i>	3002	nitrotriacetate monooxygenase	<i>yqcD</i>	2667	phage-related protein
<i>gsiB</i>	494	general stress protein	<i>yubB</i>	3195	bactitracin resistance protein (undecaprenol kinase)	<i>yqXH</i>	2666	phage-related lytic exoenzyme
<i>gspA</i>	3944	general stress protein				<i>yqxG</i>	2665	holin
<i>hit</i>	1076	Hit-like protein involved in cell-cycle regulation	<i>yusi</i>	3366	arsenate reductase	IV.5	TRANSPOSON AND IS	10
<i>htgD</i>	4090	class III heat-shock protein (chaperonin)	<i>yvtT</i>	3487	alkanol monooxygenase	<i>ydcP</i>	533	transposon protein
<i>htrA</i>	1359	serine protease Do (heat-shock protein)	<i>yvpP</i>	3543	reticulone oxidase	<i>ydcQ</i>	533	transposon protein
<i>ispU</i>	1387	activation of σ^E	<i>ywch</i>	3810	monooxygenase	<i>ydcR</i>	535	transposon protein
<i>lonA</i>	2882	class III heat-shock ATP-dependent protease	<i>ywnH</i>	3760	phosphothricin acetyltransferase	<i>ydcB</i>	537	transposon protein
<i>lonB</i>	2884	Lon-like ATP-dependent protease	<i>yxel</i>	4062	penicillin amidase	<i>ydeE</i>	538	transposon protein
<i>mrqA</i>	3983	metallorepression DNA-binding stress protein	<i>yxeK</i>	4061	monooxygenase	<i>yddH</i>	544	transposon protein
<i>rsbR</i>	519	positive regulator of σ^E activity (interaction with RsbS)	<i>yysR</i>	4185	streptothricin acetyl-transferase	<i>yefB</i>	739	site-specific recombinase
<i>rsbS</i>	520	negative regulator of σ^E activity (antagonist of RsbT)	IV.3	ANTIBIOTIC PRODUCTION	30	<i>yefC</i>	739	resolvase
<i>rsbT</i>	520	positive regulator of σ^E activity (switch protein/serine kinase [RsbS])	<i>pksB</i>	1782	involved in polyketide synthesis	<i>yneB</i>	1918	resolvase
<i>rsbU</i>	521	indirect positive regulator of σ^E activity (serine phosphatase [RsbV-P])	<i>pksC</i>	1783	involved in polyketide synthesis	<i>yocA</i>	2085	transposon-related protein
<i>rsbV</i>	522	positive regulator of σ^E activity (anti-anti-sigma factor [RsbV])	<i>pksD</i>	1785	involved in polyketide synthesis	IV.6	MISCELLANEOUS	26
<i>rsbW</i>	522	negative regulator of σ^E activity (switch protein/serine kinase [RsbV], anti-sigma factor [σ^E])	<i>pksE</i>	1785	involved in polyketide synthesis	<i>bex</i>	2610	GTP-binding protein
<i>rsbX</i>	523	indirect negative regulator of σ^E activity (serine phosphatase [RsbS-P])	<i>pksF</i>	1788	involved in polyketide synthesis	<i>csbA</i>	3614	putative membrane protein
<i>ycdH</i>	308	adhesion protein	<i>pksG</i>	1789	involved in polyketide synthesis	<i>csbB</i>	36	σ^E -transcribed gene
<i>ydaG</i>	473	general stress protein	<i>pksH</i>	1790	involved in polyketide synthesis	<i>ctaG</i>	1564	function unknown
<i>yfjL</i>	910	surface adhesion	<i>pksI</i>	1791	involved in polyketide synthesis	<i>eag</i>	1430	small membrane protein
<i>yfjR</i>	914	heat-shock protein	<i>pksJ</i>	1792	involved in polyketide synthesis	<i>ecsC</i>	1079	function unknown
<i>ykZA</i>	1381	general stress protein	<i>pksK</i>	1794	polyketide synthase	<i>mmgE</i>	2509	function unknown
<i>yloA</i>	1637	fibrinectin-binding protein	<i>pksL</i>	1808	polyketide synthase	<i>nifZ</i>	3027	NiF protein homologue
<i>yloU</i>	1655	alkaline-shock protein	<i>pksM</i>	1821	polyketide synthase	<i>sapB</i>	726	mutant activates alkaline phosphatase during sporulation independently of σ^E and σ^H
<i>ynbA</i>	1875	GTP-binding protein protease modulator	<i>pksN</i>	1834	polyketide synthase	<i>sbp</i>	1595	small basic protein
<i>ynzF</i>	1880	δ -endotoxin	<i>pksP</i>	1835	polyketide synthase	<i>veg</i>	53	function unknown
<i>yocK</i>	2097	general stress protein	<i>pksR</i>	1850	polyketide synthase	<i>yael</i>	102	creatine kinase
<i>yocM</i>	2098	small heat-shock protein	<i>ppsA</i>	1997	peptide synthetase	<i>ybaL</i>	157	ATP-binding Mip-like protein
<i>yodU</i>	2151	capsular polysaccharide biosynthesis	<i>ppsB</i>	1990	peptide synthetase	<i>ybcU</i>	287	NiF protein homologue
<i>yokG</i>	2279	δ -endotoxin	<i>ppsC</i>	1982	peptide synthetase	<i>yefN</i>	730	pet112-like protein
<i>yqpP</i>	2286	capsular polysaccharide biosynthesis	<i>ppsD</i>	1974	peptide synthetase	<i>yhdP</i>	1033	hemolysin
<i>yxG</i>	3047	general stress protein	<i>ppsE</i>	1963	peptide synthetase	<i>yhdT</i>	1035	hemolysin
<i>yxH</i>	3047	general stress protein	<i>sbo</i>	3835	subtilisin A	<i>yheG</i>	1049	calcium-binding protein
<i>yxiJ</i>	3046	general stress protein	<i>sfp</i>	408	surfactin production	<i>yplQ</i>	2295	hemolysin III homologue
<i>yxeK</i>	3529	capsular polysaccharide biosynthesis	<i>srfAA</i>	377	surfactin synthetase / competence	<i>yqxG</i>	2523	hemolysin-like
<i>yxeL</i>	3528	capsular polysaccharide biosynthesis	<i>srfAB</i>	387	surfactin synthetase / competence	<i>yrfA</i>	2720	hemolysin-like
<i>yxeM</i>	3527	capsular polysaccharide biosynthesis	<i>srfAC</i>	398	surfactin synthetase / competence	<i>yrvO</i>	2811	NiF protein homologue
<i>yxeN</i>	3524	exopolysaccharide biosynthesis	<i>srfAD</i>	402	surfactin synthetase / competence	<i>yuaG</i>	3187	epidermal surface antigen
<i>yxeP</i>	3523	capsular polysaccharide biosynthesis	<i>sunA</i>	2269	subtilisin 1681 antibiotic antimicrobial precursor peptide	<i>yurV</i>	3357	NiF protein homologue
<i>yxeQ</i>	3522	capsular polysaccharide biosynthesis	<i>yomB</i>	2264	bacteriocin	<i>yurW</i>	3358	NiF protein homologue
<i>yxeR</i>	3521	spore coat polysaccharide biosynthesis	<i>yukL</i>	3282	antibiotic synthetase	<i>yutI</i>	3309	NiF protein homologue
<i>yxeT</i>	3519	capsular polysaccharide biosynthesis	<i>yukM</i>	3283	antibiotic synthetase	V	SIMILAR TO UNKNOWN PROTEINS	668
<i>yvIC</i>	3517	capsular polysaccharide biosynthesis	IV.4	PHAGE-RELATED FUNCTIONS	83	V.1	FROM <i>B. SUBTILIS</i>	177
			<i>codV</i>	1687	integrase/recombinase	V.2	FROM OTHER ORGANISMS	491
			<i>ripX</i>	2449	integrase/recombinase	VI	NO SIMILARITY	1053
			<i>xhiA</i>	1346	involved in cell lysis upon induction of PBSX			
			<i>xhiB</i>	1346	hydrolysis of 5-bromo 4-chloroindolyl phosphate upon induction of PBSX (holin)			
			<i>xxdA</i>	1320	PBSX prophage			
			<i>xxdB</i>	1321	PBSX prophage			

The optically dark side of galaxy formation

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Deep optical surveys^{1,2} probe the rest-frame ultraviolet luminosities of high-redshift galaxies. They can therefore be used to infer star formation rates, under assumptions about young stellar populations. Current data suggest that the global star-formation rate of the Universe peaked at a redshift of $z = 1$, then subsequently declined^{3,4}, leading to claims that the bulk of star formation in the Universe has been seen. However, the large uncertainties inherent in correcting for ultraviolet absorption by dust associated with young stars suggest that these formation rates might be substantially underestimated in high-redshift galaxies. Here we circumvent this problem by considering the dust thermal emission at infrared (and submillimetre) wavelengths. We propose an improved determination of the long-sought cosmic infrared background⁵ (built up from the accumulated infrared light of faint galaxies along the line of sight), from which we are able to estimate the required population of high-redshift, dust-enshrouded starburst galaxies. We argue that most of the star formation at high redshift may be hidden by dust, and we define the necessary characteristics of a feasible far-infrared survey that could detect this population.

Although only one-third of the bolometric luminosity of local galaxies is radiated in the infrared⁶, there is a growing evidence that this fraction is actually increasing with redshift. The deepest counts available from the Infrared Astronomical Satellite (IRAS) at $60\ \mu\text{m}$ (ref. 7), which correspond to an average redshift (z) of only 0.2 (ref. 8), already suggest some evolution of the infrared emission in the Universe. A recent deep survey with the Infrared Space Observatory (ISO) at $15\ \mu\text{m}$ has discovered a few objects at $z \sim 0.5$ to 1 with star formation rates much higher than deduced from the optical⁹. However, the strongest constraint on the high-redshift infrared emission is given by the cosmic infrared background (CIRB) found in data acquired by the Far-Infrared Absolute Spectrophotometer (FIRAS) instrument on board the Cosmic Background Explorer (COBE) satellite in the $200\ \mu\text{m}$ – $2\ \text{mm}$ wavelength range⁵. Several steps are necessary to remove the foreground Galactic components and extract the isotropic residual identified as the CIRB. Whereas, at the wavelengths probed by FIRAS, the interplanetary emission is small and easily removed¹⁰, the emission from interstellar dust mixed with the different gas phases of the interstellar medium is the dominant component. The spectra that correlate with the 21-cm interstellar emission of neutral hydrogen (H I) (ref. 11) along lines of sight with H I column densities $N_{\text{HI}} \leq 4.5 \times 10^{20}\ \text{atoms cm}^{-2}$ are described by a modified black-body $\nu^2 B_\nu(T)$ with $T = 17.5\ \text{K}$ (ref. 12). Part of the long-wavelength excess over this simple model increases with H I column density and can be linked to dust emission associated with ionized and molecular hydrogen. The other part is an isotropic residual that has been interpreted as the CIRB. Determination of the isotropy required the use of a large fraction of the sky⁵. This therefore involved correcting for substantial foreground components. Any inaccuracy in this correction might have contributed a spurious signal. To address this problem, the original method of Puget *et al.*⁵

was applied again, but only in the cleanest regions with very low H I column densities ($N_{\text{HI}} \leq 10^{20}\ \text{atoms cm}^{-2}$ instead of $N_{\text{HI}} \leq 4.5 \times 10^{20}\ \text{atoms cm}^{-2}$). In that case, the residual component totally dominates the emission (Fig. 1). This demonstrates that it cannot be due to artefacts in the removal of interstellar emission. Figure 1 also shows that the CIRB intensity per frequency decade $\nu I_\nu \approx 7 \times 10^{-9}\ \text{W m}^{-2}\ \text{sr}^{-1}$ near $300\ \mu\text{m}$ is a factor of 5 higher than the no-evolution prediction obtained by a simple extrapolation of the infrared luminosities of local galaxies. Its level is comparable to that of the 'cosmic optical background' estimated by summing the faint galaxy counts down to the deepest limit so far available, which is given by the Hubble Deep Field².

To break the background into the contributing sources, we need to model the infrared/submillimetre emission of galaxies. Galaxy formation and evolution can be briefly sketched as follows. Small

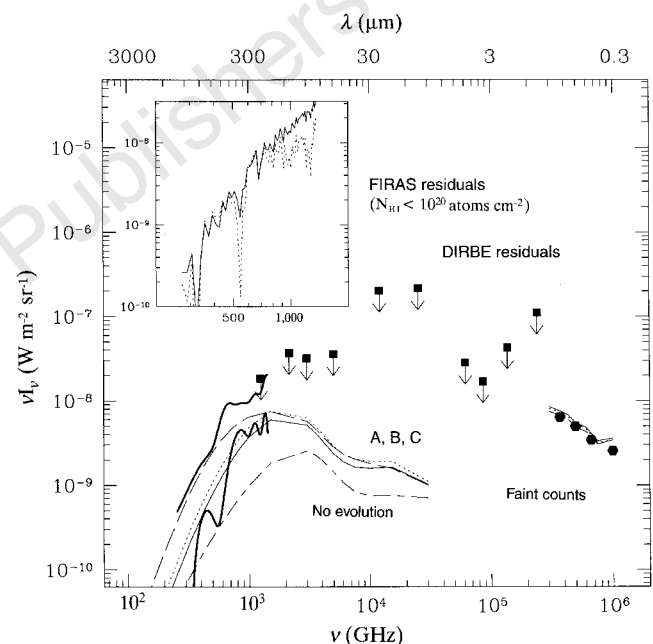


Figure 1 Inset, high-latitude COBE/FIRAS spectrum in regions with very low H I column densities ($N_{\text{HI}} \leq 10^{20}\ \text{atoms cm}^{-2}$; solid line) and residual spectrum after subtraction of emissions correlated with neutral and ionized hydrogen (dotted line). This component is likely to be the CIRB. Main panel, the $\pm 1\sigma$ error bars per point have been used to define an acceptable range for CIRB predictions (thick solid lines). Solid squares show the upper limits given by COBE/DIRBE residuals²⁶. The solid hexagons show the cosmic optical background obtained by summing up faint galaxy counts down to the Hubble Deep Field limit. Strictly, this is only a lower limit of the actual optical background, but the shallowing of the U and B -band counts suggests near-convergence at least at those wavelengths². The dot-dashed line shows the background predicted without evolution, in a universe with $H_0 = 50\ \text{km s}^{-1}\ \text{Mpc}^{-1}$ and $\Omega_0 = 1$. The solid, dotted and broken lines show the predictions for our models A, B, and C, respectively, in the standard CDM model ($H_0 = 50\ \text{km s}^{-1}\ \text{Mpc}^{-1}$, $\Omega_0 = 1$, $\Lambda = 0$, $\Omega_b = 0.05$ and $\sigma_8 = 0.67$). Stars form in cold cores according to Salpeter's stellar initial mass function (with slope $s = 1.35$, and lower and upper mass cut-offs 0.1 and $120\ M_\odot$). Star formation rates are proportional to the cold gas contents, with characteristic time scales derived from the core dynamical times as $t_* = \beta t_{\text{dyn}}$. Model A has a mix of two populations, a disk-like one with $\beta = 100$, and starbursts with $\beta = 10$. The mass fraction involved in bursts increases with the formation redshift z_{for} according to $f_{\text{burst}} \propto (1 + z_{\text{for}})^5$, as suggested by the increasing fraction of pairs seen at larger z (refs 20–22). In models B and C we add a population of ULIRGs in which all the energy available from stellar nucleosynthesis ($0.007\ \text{MeV}$) is radiated by massive stars ($\langle \kappa \rangle = 0.40$) in a heavily extinguished medium. Model B has a 5% constant mass fraction of ULIRGs at all z_{for} . In model C, 90% of all galaxies forming at $z_{\text{for}} \geq 3.5$ are ULIRGs.

fluctuations in the high- z Universe grow by gravitational instability until they form dense clumps where the baryonic gas is collisionally heated. Where the density and temperature are appropriate, gas cools by emitting radiation, and the baryons pile up in cold cores whose final radii are set by angular-momentum conservation. Simultaneously, larger clumps form and encompass/accrete the previous generation of small clumps. Stars form in the cores and enrich the primordial gas via supernova ejecta. Part of the starlight is absorbed by various dust components which re-radiate at longer wavelengths according to characteristic infrared spectra. The so-called 'semi-analytic modelling' of these series of physical processes has been quite successful in reproducing the overall properties of galaxies in the optical range^{13,14}. We have elaborated an extension of this method to the infrared/submillimetre range. Details of the modelling, and complementary predictions, will be given elsewhere¹⁵.

Specifically, we assume a standard Cold Dark Matter cosmological model¹⁶ with a Hubble constant $H_0 = 50 \text{ km s}^{-1} \text{ Mpc}^{-1}$, a density parameter $\Omega_0 = 1$, a cosmological constant $\Lambda = 0$, a baryon fraction $\Omega_b = 0.05$, and a normalization $\sigma_8 = 0.67$ for the power spectrum of linear fluctuations. The sensitivity of semi-analytic modelling to cosmological parameters is known to be weak¹⁷. In our reference model A, we consider a mix of two broad types of population, one with low star formation rates (which reproduce the observational distribution of gas consumption time

scales t_* in disk galaxies¹⁸), the other proceeding in bursts with star formation rates ten times larger. We assume that bursts are triggered by interaction and merging of sub-galactic clumps¹⁹ and increase with z according to the fraction of pairs^{20–22}. This population of 'mild starbursts' and 'luminous ultraviolet/infrared galaxies' (similar to IRAS 'luminous infrared galaxies'²³) dominate the optical background (Fig. 1). The infrared luminosity-to-mass ratio is $L_{\text{IR}}/M = 6L_{\text{bol}}/M_{\odot}$ for a typical starburst with $t_* = 0.33 \text{ Gyr}$. The range of derived infrared-to-blue luminosity ratios is characteristic of blue-band selected samples²⁴ such as the Canada–France Redshift Survey (selected in the observer-frame I_{AB} band, roughly corresponding to the B band at $z \sim 1$), or the high- z galaxies of the Hubble Deep Field. Figure 1 also displays the predicted infrared background, which is clearly barely compatible with the observed CIRB, whose mean amplitude is twice higher.

To assess how much star formation might be completely hidden by dust shrouds, we consider an additional population, similar to IRAS 'ultra-luminous infrared galaxies' ('ULIRGs')²³. We maximize their infrared luminosities by assuming that all the energy available from stellar nucleosynthesis is radiated by massive stars and heats up the dust. As a consequence of a stellar initial mass function with short-lived, massive stars, the post-starburst phase is 'dark' and would be detectable only by its nucleosynthesis products. The luminosity-to-mass ratio now is $L_{\text{IR}}/M = 130L_{\text{bol}}/M_{\odot}$ for a typical starburst with $t_* = 0.33 \text{ Gyr}$. Our models B and C respectively

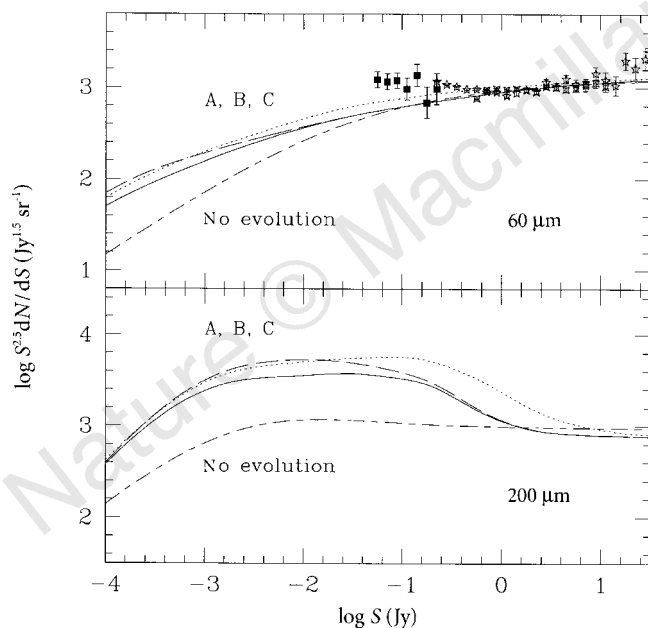


Figure 2 Predictions for differential counts normalized to Euclidean counts at $60 \mu\text{m}$ (upper panel) and $200 \mu\text{m}$ (lower panel). The dot-dashed line shows the predicted counts without evolution, in a universe with $H_0 = 50 \text{ km s}^{-1} \text{ Mpc}^{-1}$ and $\Omega_0 = 1$. The solid, dotted and broken lines show the predictions for our models A, B and C, respectively in the SCDM model (see the text). Data are plotted for IRAS counts at $60 \mu\text{m}$: open stars, Faint Source Survey²⁶; open squares, QMW survey²⁷; solid squares, North Ecliptic Pole Region⁷. The IRAS $60 \mu\text{m}$ counts suggest some evolution, but do not discriminate between the various models. These models also predict a $60 \mu\text{m}$ background fluctuation per beam in the Very Faint Source Survey (after removal of $\geq 4\sigma_{\text{tot}} = 120 \text{ mJy}$ sources) at the level of 14.1 mJy (A), 16.0 mJy (B) and 14.3 mJy (C), whereas the measured 68% quantile is $30.1 \pm 1.2 \text{ mJy}$ (ref. 28). With a 25 mJy r.m.s. instrumental noise and 6.5 mJy r.m.s. cirrus fluctuations²⁹, there is still space for a $15.4^{+2.2}_{-2.5} \text{ mJy}$ fluctuation due to sources, in good agreement with our estimates. In contrast, models A, B and C predict much stronger evolution at $200 \mu\text{m}$. The differences in the predicted counts originate from the differences in the high-redshift IR emissions.

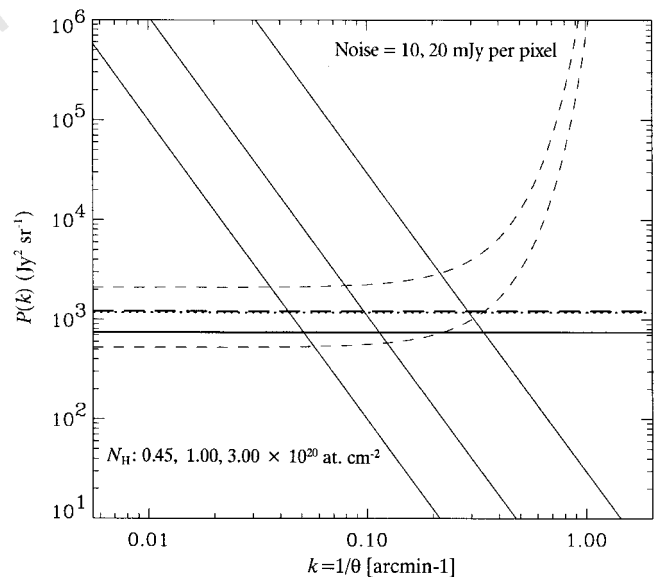


Figure 3 Comparison of predicted power spectra for observations in the ISO C160 filter. The straight horizontal lines (same code as in Fig. 1) show the predicted background fluctuations at the confusion limit (that is, its r.m.s. value equal one-third of the limiting flux of all resolved and removed sources). The beam was modelled as a gaussian with 1 arcmin full width at half maximum. The cirrus fluctuations (thin straight lines) are described by a k^{-3} power law²⁹ with levels (from left to right) set by column densities of H I typical of the Lockman hole (minimum, 0.24% of the sky), and of 4.6% and 21.8% of the sky, respectively. The detector noise spectrum of fluctuations (thin broken lines), assumed to be white, is shown for a sensitivity level of 10 and 20 mJy per 1.5 arcmin pixel. To allow a direct comparison, this flat white noise has been projected on the sky, that is, divided by the Fourier transform of the beam profile. The resulting exponential rise of the on-sky noise thus bounds the range of available scales. The comparison shows that, in the cleanest regions of the sky (for example, $N_{\text{H I}} = 10^{20} \text{ atoms cm}^{-2}$), unresolved background fluctuations for models B and C actually dominate both cirrus and noise fluctuations at scales $3 < \theta < 10 \text{ arcmin}$, if the r.m.s. noise level is 10 mJy per pixel.

mimic continuous bulge formation as the end-product of interaction and merging, and a strong episode of bulge formation at $z_{\text{for}} > 3.5$. Both are consistent with the observed CIRB. The high-redshift, dust-enshrouded, star formation in model C results in a high level of the predicted background at wavelengths $\lambda > 400 \mu\text{m}$.

Although none of the currently available optical data reflect the large differences between these models, which originate in the different fractions of heavily extinguished objects, the predicted infrared/submillimetre counts are more interesting, as shown in Fig. 2. Comparison of IRAS data with the no-evolution curve at $60 \mu\text{m}$ suggests some evolution. However, it seems that the $60\text{-}\mu\text{m}$ band does not strongly discriminate between the various models of evolution. In contrast, the upward deviation at $200 \mu\text{m}$ is due to the contribution of the redshifted $100\text{-}\mu\text{m}$ maximum of the infrared energy distribution. This redshifting of steep spectra counterbalances distance dimming and can make high- z objects easier to detect than low- z ones. Submillimetre observations are thus quite sensitive to the high- z history. The model also predicts that, at $200 \mu\text{m}$, $10\text{--}100\text{-mJy}$ sources (contributing to 15% of the background) are located mostly at $z \sim 0.5\text{--}2.5$, whereas at $60 \mu\text{m}$, and at the typical sensitivity level of IRAS surveys, the sources are indeed located mostly at very low z .

The detection of these sources would be a strong test for assessing the level of the 'optically dark' side of galaxy formation. The C160 filter of the ISOPHOT instrument on board ISO has an effective wavelength $\lambda_{\text{eff}} \approx 175 \mu\text{m}$ for typical spectra of distant galaxies, and a 10 mJy r.m.s. noise fluctuation per 1.5 arcmin pixel is reachable after integration times longer than $\sim 256 \text{ s}$ per pixel. Thus a deep survey with this instrument seems to be feasible and is indeed scheduled. However, one might be concerned that small-scale cirrus fluctuations could hide the sources and the background fluctuations that they induce. A comparative analysis of the expected power spectra due to (1) cirrus fluctuations in regions of various H I column densities, (2) background fluctuations once sources above the confusion limit have been removed, and (3) the detector noise, shows that, in clean regions of the sky ($N_{\text{HI}} \leq 10^{20} \text{ atoms cm}^{-2}$), a survey with 10 mJy r.m.s. sensitivity should not only detect most sources above the low cirrus fluctuations but could also see background fluctuations in excess of the detector noise fluctuations, on scales of $3\text{--}10 \text{ arcmin}$ (see Fig. 3). Because model C has 6.3×10^5 sources per sr with fluxes $> 30 \text{ mJy}$, a deep survey of a $\sim 1,000 \text{ arcmin}^2$ field might begin to 'break' the CIRB into $\sim 50 \pm 7$ discrete sources, an order of magnitude more than is expected without evolution. This number is sufficient to test the high level of evolution and even to begin to disentangle the various models. If detected, the level of background fluctuations can also help to constrain the redshift distribution of the sources.

The tentative discovery of the CIRB is now supported by our new study in the cleanest regions of the sky, where the foreground Galactic components are essentially negligible. The conversion of the CIRB into its contributing sources by means of a semi-analytic model of galaxy formation leads to predictions of faint galaxy counts at infrared and submillimetre wavelengths. In contrast with the status of IRAS $60\text{-}\mu\text{m}$ counts, $175\text{-}\mu\text{m}$ counts with ISO should be able to detect the predicted strong evolution, and even disentangle various models consistent with the level of the CIRB. Moreover, small-scale cirrus fluctuations cannot hide the presence of the sources. Consequently, our knowledge of the optical/infrared luminosity budget at $z \sim 0.5\text{--}2.5$ should improve rapidly. We are about to start unveiling the optically dark side of galaxy formation. $\square$

Received 15 May; accepted 2 October 1997.

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Acknowledgements. We thank Dave Clements, François-Xavier Désert and Bruno Maffei for their comments and suggestions.

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Phase transitions in individual sub-micrometre superconductors

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The properties of a superconductor are expected to change radically when its size becomes comparable to that of the Cooper pairs, the quasiparticles responsible for superconductivity. The effect of such confinement is well understood for the case of the suppression of superconductivity by magnetic fields (which gives rise to so-called Little–Parks oscillations of the phase boundary)^{1–4}. But little is known about what happens in small superconductors in the zero-resistance state, which cannot be

probed by resistance measurements. Here we apply a new technique of ballistic Hall magnetometry⁵ to study the magnetization of individual superconducting discs of diameters down to 100 nm. The superconducting state of these discs is found to be qualitatively different from both macroscopic and microscopic⁶ superconductors, with numerous phase transitions whose character changes rapidly with size and temperature. This exotic behaviour is due to size quantization of the Cooper-pair motion and resulting transitions between discrete states of the superconducting Bose condensate in a magnetic field.

The oscillating superconducting phase boundary of small hollow cylinders² and discs^{3,4} (the Little–Parks effect) implies that spatial confinement should also essentially change the superconducting state of samples whose size is comparable to the characteristic size of Cooper pairs (coherence length ξ)¹ and the superconductivity of which is destroyed by only a few magnetic flux quanta. Although the Little–Parks effect has been known for almost 40 years, it has so far proved to be impossible to conduct analogous studies inside the superconducting state, mainly because the commonly used resistance measurements are unsuitable as the resistance remains zero even if there are changes occurring below the superconducting transition temperature (T_C). A technique recently developed by our group and referred to as ballistic Hall micro-magnetometry⁵ allows us to perform thermodynamic studies of individual superconducting grains and thus to probe their superconducting state below T_C . The technique enables the detection of magnetization changes of $\sim 10^3$ Bohr magnetons ($\sim 10^{-17}$ emu) and its details are briefly described in the legend to Fig. 1. The smallest sample from which we were able to get a reliable superconducting response was only ~ 100 nm in size and even this limit is mainly due to the absence of the Meissner effect in such small particles^{1,6}.

Figure 2 shows several magnetization curves that demonstrate a surprising diversity of behaviour in mesoscopic superconductors: a

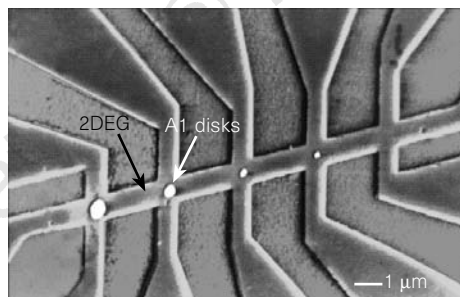


Figure 1 Scanning electron micrograph (viewed at an angle) of our experimental devices. It shows five Hall probes made from GaAlAs/GaAs heterostructures with a two-dimensional electron gas (2DEG) embedded 60 nm below the surface. Superconducting Al discs of various diameters are deposited in the probes' sensitive areas and electrically insulated from the 2DEG. Ballistic Hall magnetometry is based on precise measurements of the magnetic field in the direct vicinity of a mesoscopic sample placed in the central area of a sub-micrometre Hall probe. Owing to a relatively low electron concentration, a 2DEG provides large, easily measurable Hall response. However, the main reason for using a 2DEG is that electrons move ballistically inside the cross junction. For ballistic motion, the Hall voltage is simply given by the average magnetic field in the cross junction, that is, $R_{xy} \propto \langle B \rangle / ne$, thus allowing straightforward quantitative analysis. Because the magnetic flux inside the cross junction is $\Phi = \langle B \rangle S$, these ballistic Hall probes are in fact micro-fluxmeters, similar to SQUIDs but with an effective detection loop of only $\sim 1 \mu\text{m}^2$. The difference between the Hall signal for the probes with and without a superconducting sample gives directly the magnetization $4\pi M = \langle B \rangle - H$.

change of only a factor of two in the size of sub-micrometre aluminium discs drastically changes their superconducting response. The smallest sample of Fig. 2a and smaller discs exhibit a simple magnetization response, with the second-order phase transition between the normal and superconducting states (smooth decay of magnetization). Such behaviour is the same as that expected for thin films in parallel field and is in agreement with theory¹. For the sample of twice the size, the superconducting phase transition unexpectedly becomes first order (as indicated by a jump in magnetization) and is accompanied by a well-defined bistable region where the sample can be either superconducting or normal, depending on the direction of the magnetic sweep. Both states can live for several hours (so it was not possible to determine which one was thermodynamically stable) and were found only for a very narrow range of sizes ($r \approx \xi(0)$) and at temperatures far below T_C . When the sample size was increased further, we found that the superconducting phase transition became second order again. In addition, numerous magnetization jumps appeared, indicating first-order phase transitions inside the superconducting state. Although the magnetization $M(H)$ depends on the direction of the field sweep, the remanent magnetization always remains zero, indicating the absence of detectable bulk pinning. The hysteresis in $M(H)$ is due to the surface (Bean–Livingston) barrier.

The initial stages of magnetization of the large discs (Fig. 2c, d) can be understood qualitatively as an entry or exit of individual vortices into or out of the superconductor. Indeed, although the height of the magnetization jumps here is not quantized, they are all of about one flux quantum, ϕ_0 . However, in higher fields and for the

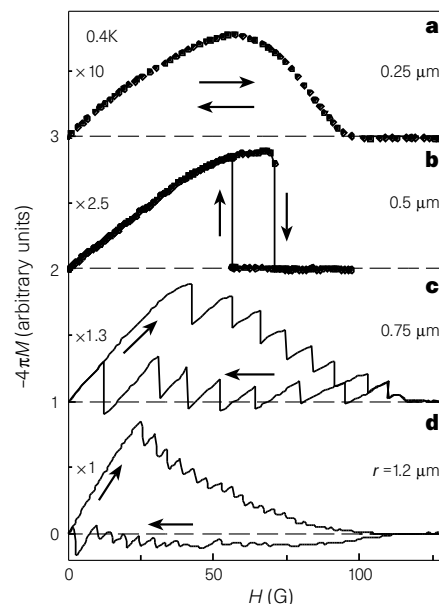


Figure 2 Magnetization responses of individual sub-micrometre Al discs of various radii. Radii are 0.25 (a), 0.50 (b), 0.75 (c) and 1.20 μm (d). The curves are reproducible within the line width, even after thermal cycling to room temperature. Arrows show the direction of the magnetic field sweep. Aluminium is a favourable material for studies of mesoscopic superconductivity owing to its large coherence length $\xi(T = 0\text{ K})$, which can be of the order of $1 \mu\text{m}$ even in evaporated thin films with typically short electron mean free path l . The resistivity of our macroscopic Al films yields an l of ~ 50 nm, varying by a factor of 2 depending on the film thickness and evaporation conditions. T_C is ~ 1.3 K. This allows us to estimate the superconducting parameters¹: $\xi(0\text{ K}) \approx 250$ nm and $\lambda(0) \approx 70$ nm; that is, in the bulk our material is a type-I superconductor. We have measured the magnetization of many discs with radii r of 70 nm to 1.3 μm and heights of 70 nm to 0.15 μm . The curves presented are for height 0.13 μm , but no qualitative difference in the behaviour is found for the other heights.

largest samples (as in Fig. 2d) the flux jumps become only a small fraction of ϕ_0 and eventually disappear in the noise. This observation is in striking contrast with the common belief that the field penetrates in superconductors in units of ϕ_0 . There are also several other peculiar features on the magnetization curves (for example, smooth penetration of magnetic field between flux jumps) that will be discussed elsewhere.

The drastic changes in the superconducting response with changes in sample size can be understood qualitatively as entering three different regimes with respect to the size of Cooper pairs: $r \leq \xi$ (upper curve), $r \approx \xi$ (middle) and $r > \xi$ (two lower curves). Alternatively, $r \approx \xi$ implies $H_{c2}S/\phi_0 \approx 1$, where $H_{c2} = \phi_0/2\pi\xi^2$ is the critical field¹, and our mesoscopic superconductors can also be described as 'few-flux-quanta' superconductors in which the superconductivity is destroyed by only a few vortices. The parameter that is expected to determine the observed behaviour is the relative size of the sample, r/ξ . Indeed, because $\xi(T) \approx \xi(0)[1 - (T/T_C)]^{-1/2}$ (ref. 1), we could move between the various regimes by varying the temperature, for example the disc with $r = 0.5 \mu\text{m}$ at $T < 0.8 \text{ K}$ exhibits an $M(H)$ similar to that for the $0.25 \mu\text{m}$ disc at 0.4 K . Although for most experimental features there is good scaling between different temperatures and sizes, the bistable region was never observed above 0.8 K .

To understand the unexpected bistable superconductivity, we have measured the superconducting phase boundary for several samples in the regime $r \approx \xi(0)$. Figure 3 shows $H_{cr}(T)$ for the 'two-flux-quanta' superconductor and, for comparison, $H_{cr}(T)$ of a macroscopic aluminium film deposited simultaneously with the disc. All the difference between the two diagrams stems from the presence of the sample boundary. The diagram for the small disc exhibits an arc-type behaviour that at temperatures close to T_C is known as Little–Parks oscillations (at lower temperatures these oscillations were previously not accessible)^{2–4}. We find that the arcs

continue into lower temperatures where the bistable region appears as a splitting into two branches that starts at precisely the same temperature as that at which the superconducting transition changes its order.

For a quantitative analysis of the observed phase diagrams, we use the linearized Ginzburg–Landau (GL) equation^{1–4}. As demonstrated in Fig. 3, it describes the phase diagram of the mesoscopic disc perfectly, with no fitting parameters (ξ is determined experimentally from the reference film). The different arcs in Fig. 3 correspond to quantum states (solutions of the GL equation) with different orbital momenta L . They describe different distributions of the superconducting current in the disc and can be viewed as giant multi-quanta vortices⁴ (Fig. 3, insets). The overall increase in H_{cr} in the mesoscopic sample relative to the film is by a factor of ~ 1.7 and is obviously related to the surface superconductivity ($H_{c3} = 1.69H_{c2}$) (ref. 1).

Surprisingly, the curves in Fig. 3 describe the phase boundary accurately even at low T , although GL equations are generally no longer valid in this temperature interval¹. The theoretical fit imparts a very clear physical meaning to the bistable region in Fig. 3: the latter corresponds to the transition between the ground ($L = 2$) and first excited ($L = 1$) states of the superconducting condensate. The quantized bistability is bound to low temperatures but not to any particular value of L , because for other samples (with slightly different r/ξ) it has also been observed for transitions between $L = 0$ and 1 and between $L = 2$ and 3. The bistability suggests the presence of a potential barrier between different L states, whereas its absence at temperatures close to T_C probably indicates that it is destroyed by thermal activation above the barrier.

Now we turn to the discussion of the transitions observed inside the superconducting state of larger discs (Fig. 2c). The magnetization jumps unambiguously indicate first-order phase transitions between discrete thermodynamic states of the superconducting

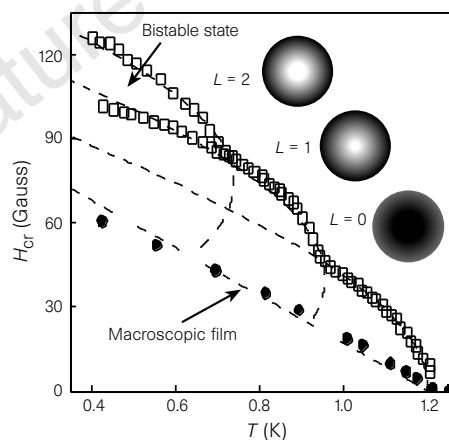


Figure 3 Typical superconducting phase diagram observed for mesoscopic disks with $r \approx \xi(0)$. The critical field H_{cr} is measured as the onset of the magnetization response at different temperatures. The squares show the diagram for a $0.55\text{-}\mu\text{m}$ Al disc (thickness $0.08 \mu\text{m}$); the ovals are for the reference Al film. The broken curves show the corresponding solutions of the linearized GL equation. For the macroscopic film, the equation yields the known expression $H_{cr}(T) = \phi_0/2\pi\xi(T)^2 = \phi_0/2\pi\xi(0)^2(1 - T/T_C)$ (ref. 1), which describes the experimental temperature dependence well. The fit yields $\xi(0) \approx 0.2 \mu\text{m}$, in agreement with the value found independently from the film resistivity. The calculated distribution of the order parameter for different states L is shown as insets: black corresponds to the maximum of the order parameter, and white to its minimum.

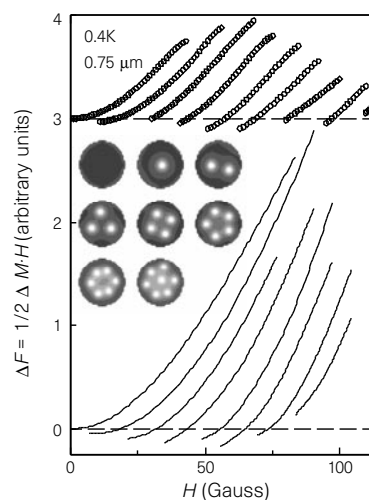


Figure 4 Energy of the superconducting states observed in the $0.75 \mu\text{m}$ disc of Fig. 2 plotted against magnetic field at 0.4 K (top). The magnetization data are converted into energy by using the equation $\Delta F = M^*H/2$. The data were obtained by sweeping the magnetic field up and down, starting at different fields, so that all possible states of the mesoscopic system could be scanned. The superconductor can evolve along any of the curves until it becomes unstable and switches to the next state either by the entry or the exit of a vortex. The stability range is defined by the surface barrier. Switch-overs between different states (jumps in the magnetization) are omitted from the plot. The lower part of the figure shows numerical simulation of our experiment with the full set of two-dimensional GL equations. The large range of stability on the theoretical curves is due to the cylindrical geometry used in the simulations, which ignores demagnetization effects.

condensate. The dependence of the energy of these states on the magnetic field is plotted in Fig. 4 (top). One could predict that such states are due to different numbers of Abrikosov vortices that can fit into a small volume. The situation resembles magnetization peaks observed in layered superconductors as the result of varying numbers of vortex rows in the sample^{7,8}. The initial stages of magnetization in mesoscopic superconductors were also described as evolution along different vortex phases in recent theoretical works, using a semi-classical model of interacting vortices^{9,10}. We have obtained a better and more complete description of the experimental data by numerically solving the nonlinear GL equations^{1,8}; the results are shown in Fig. 4. The quantum states clearly correspond to integral numbers N of Abrikosov vortices (Fig. 4, insets). The different curves $\Delta F(H)$ are shifted along the horizontal axis by $\Delta H \approx \phi_0/s$, indicating one-by-one entries of vortex cores inside the disk area s . Note that although the number of vortices in the sample is quantized, there is no quantization of the magnetic flux because some of it 'spills out' of the sample owing to the presence of the boundary. This also leads to smooth penetration of the magnetic field inside the superconductor (nonlinear Meissner effect), which can be seen as the rounding of the upper parts of the curves in Figs 2 and 4.

Although the presence of the sample boundary explains the non-quantized flux jumps qualitatively, neither of the existing theories predicts the possibility of fractional jumps (much smaller than ϕ_0) observed in our largest, multi-flux-quanta superconductors in high fields. We attribute them to changes in the vortex configurations for a constant number of vortices inside the superconductor. Indeed, our numerical simulations of the GL equations find multiple stable vortex configurations for $N \geq 5$ (vortex configurations are also discussed in refs 8, 10). Different configurations assume different energies and lead to different amounts of magnetic flux spilling out. Therefore, transitions between such states occur with minor flux jumps. The number of possible vortex configurations increases with increasing N , which can explain the jumps getting smaller with increasing field. It is also possible that shape irregularities allow additional vortex configurations.

Finally, there is a clear analogy between our work and current intensive studies of electron spectra in confined geometries, commonly referred to as quantum dots or artificial atoms (reviewed in ref. 11). In fact, the GL equations can be seen physically as a Schrödinger-type equation for the centre-of-mass motion of Cooper pairs (with strong non-local interaction via vector potential)¹. From this viewpoint, mesoscopic superconductors present "artificial atoms made from Cooper pairs rather than electrons" and we can equally interpret Figs 3 and 4 in terms of energy spectra of non-interacting and interacting Cooper pairs, respectively. □

Received 8 May; accepted 23 September 1997.

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Acknowledgements. We thank INTAS, FOM and NATO for financial support.

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Giant fluctuations in a free diffusion process

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Macroscopic concentration gradients in physical systems relax towards equilibrium by diffusion^{1–3}, in the absence of bulk motion. This is normally regarded as a spatially homogeneous mixing process. Here, however, we show that unexpectedly large spatial fluctuations in concentration can occur during a free diffusion process. We set up an initially sharp interface between two miscible fluids by letting a mixture phase-separate below the critical consolution temperature and then raising the temperature quickly to the single-phase region. Shadowgraph images and low-angle light scattering show evidence for large fluctuations in composition, orders of magnitude larger in amplitude than those seen in the equilibrium state. We show that these pronounced inhomogeneities are due to a coupling between velocity

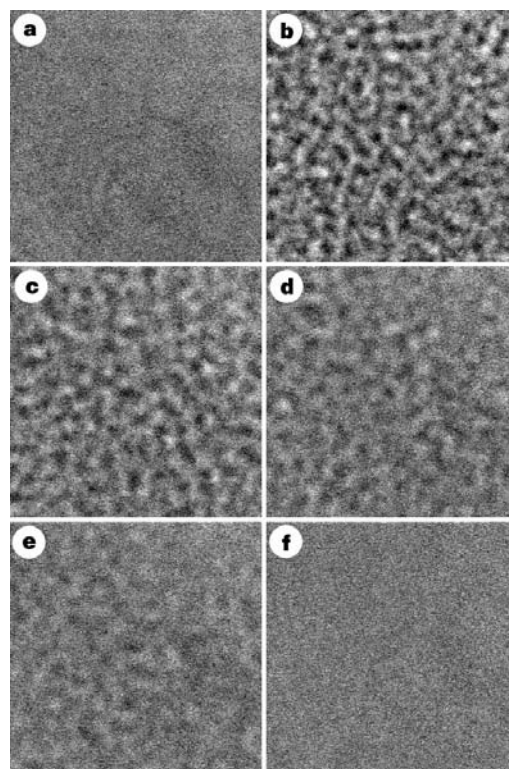


Figure 1 Shadowgraph images of the sample during the diffusion process. The images were generated by shining a vertical white-light beam on an horizontal layer of sample and by imaging the sample slightly out of focus on a CCD (charge-coupled device) camera. Image **a** was acquired when the sample was kept below its critical temperature T_c , where the sample consists of two layers of immiscible fluids separated by a sharp interface, and no diffusion occurs. A temperature jump was then applied to bring the sample above T_c , where the two layers become miscible, and the diffusion process started. The images **b–f** were taken at different times after the application of the temperature jump: **b**, 100 s; **c**, 9 min; **d**, 90 min; **e**, 330 min; and **f**, 3 days. To get rid of non-uniform illumination, the images were processed by subtracting a reference image acquired when the sample was homogeneous. The side of each image is 1 mm in real space.

and concentration fluctuations in the non-equilibrium state⁴⁻⁶. Gravity cuts off the fluctuations above a certain wavelength, and the amplitude of the fluctuations at longer wavelengths does not depend on any relevant thermodynamic property of the fluid. As a consequence, these giant fluctuations should be observable in any mixture undergoing mixing by diffusion.

In an ideal free diffusion experiment, two samples of miscible fluids at different concentrations are separated by a sharp, flat surface at time $t = 0$. A measurement of the time evolution of the concentration profiles readily gives an estimate of the diffusion coefficient^{1,2}. To avoid difficulties in the preparation of the initial sharp boundary we have chosen a liquid mixture not far from a consolution critical point (aniline and cyclohexane), the concentration being very close to the critical one⁷. The sample is first brought to a temperature T below the critical point ($T_c - T = 3$ K), so that it separates into two phases with a mass fraction concentration difference⁸ $\delta c = 0.5$, and a sharp horizontal interface is formed at the mid-height of the cell. The temperature is then rapidly brought above the critical temperature at $T - T_c = 1.0$ K. The thermal time constant is of the order of one minute, to be compared with the mass diffusion constant, which is of the order of a few hours. The large difference in the time constants is partly a consequence of working close to a critical point, as diffusion is (in our conditions) a factor of ten slower than in ordinary liquid mixtures. It must be stressed that once the new temperature above T_c has been reached, there is no phase transition to speak of, as we are dealing now with an isothermal system above T_c , which happens to be in a spatially inhomogeneous state and hence has to go back to the homogeneous state via diffusion (no remixing by convection can be expected, as the denser fluid is at the bottom).

The liquid sample is a circular, horizontal slab 38 mm in diameter and 4.50 mm thick. It is confined between two thick sapphire plates (chosen for their relatively good thermal conductivity), and the temperature is controlled to better than 1 mK. A 5 mK stabilizing temperature difference between top and bottom plates is maintained to discourage stray convection across the slab. In the set-ups

for both shadowgraphs and light scattering, the optical axis is vertical.

An image-forming white-light shadowgraph⁹ was used to form images of the fluid slab. We show in Fig. 1 a sequence of pictures obtained in the two-phase situation below T_c and at various intervals from seconds to days after the temperature jump above T_c . Regular and uniform fluctuations appear shortly after the temperature jump into the one-phase region and they decrease in amplitude as time goes on, as shown by the loss of contrast. Eventually, the system falls back to an optically homogeneous state, similar to that before the temperature jump (see Fig. 1a, f). When observed live, the patterns fluctuate with a typical time constant of the order of one second. Although largely qualitative in nature, the shadowgraph images give an estimate of the spatial extent of the fluctuations, which grow to a macroscopic level. A characteristic length is about 70 μm (Fig. 1b).

Static light scattering was used to determine the amplitude of the concentration fluctuations as a function of the scattering wave-vector $q = (4\pi/\lambda)n \sin(\theta/2)$, where θ is the scattering angle, λ is the wavelength of the probe beam and n is the refractive index of the mixture. We present data obtained with an ultra-low-angle light-scattering apparatus¹⁰, which covers a range of angles $2' < \theta < 3^\circ$, corresponding to a q -vector range $84 \text{ cm}^{-1} < q < 7,850 \text{ cm}^{-1}$.

We show in Fig. 2 a sequence of scattered intensity distributions as a function of q , taken at various times after the beginning of the diffusion process. The strongest scattering occurs for the first set (circles in Fig. 2) obtained immediately after the quench into the one-phase region. The scattered intensity diverges as q is reduced, but the curves eventually saturate at a constant value at smaller q . We have also plotted (dashed line) the scattered intensity from equilibrium fluctuations, $I_{\text{eq}} \propto (\partial n/\partial c)^2 (\partial c/\partial \mu)$, is the chemical potential as estimated from turbidity measurements¹¹ and according to the procedure explained in ref. 12. A substantial enhancement of the fluctuations by a few orders of magnitude can be clearly seen (despite this, however, the integrated effect leads to small changes in turbidity¹³).

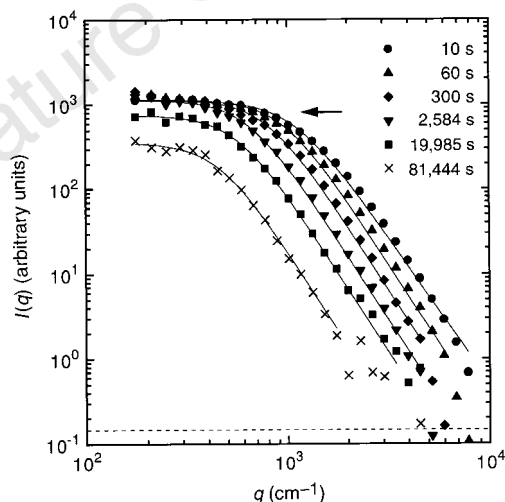


Figure 2 Non-equilibrium scattered intensity distributions plotted as a function of the wavevector q at different times during the diffusion process. The values for the elapsed time have been corrected to account for the finite time required to establish a uniform temperature across the cell (roughly 80 s). The solid line through the data is their best fit with equation 1. The dashed line is the reference equilibrium intensity scattered by the sample in its homogeneous state at $T - T_c = 1$ K. The turbidity originated from equilibrium fluctuations corresponds¹¹ to $\tau \approx 5 \times 10^{-2} \text{ cm}^{-1}$. The arrow marks the level of the non-equilibrium forward-scattered intensity as calculated from equation 3.

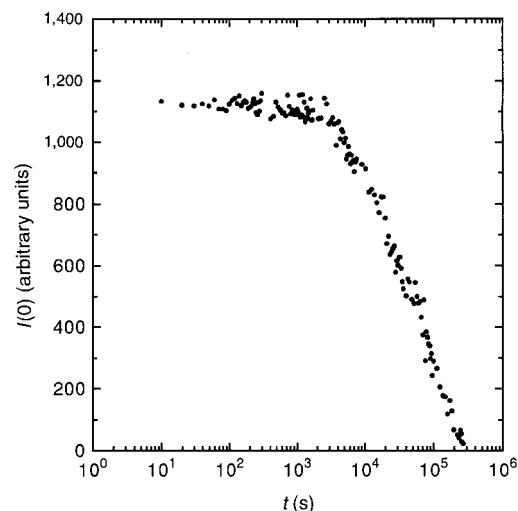


Figure 3 Forward-scattered intensity as a function of the time elapsed after the start of the diffusion process. The sudden drop in $I(0)$, which occurs for $t \approx 4,000$ s, corresponds to the transition from free diffusion behaviour (where the concentration profile evolves as if the sample were infinite in the vertical direction) to the restricted diffusion behaviour (the presence of impermeable boundaries influences the evolution of the concentration profile).

Theories of fluctuating hydrodynamics¹⁴ have been developed in connection with steady-state non-equilibrium fluctuations^{4–6,15,16}. Experimental data verifying these theories have been presented previously^{12,17–20}. The calculations of ref. 16 can be extended (A.V. and M.G., manuscript in preparation) to treat the time-dependent case of free diffusion and to calculate the scattered intensity distribution from an isothermal layer of fluid characterized by a concentration gradient ∇c . The non-equilibrium scattered intensity is given by

$$I(q, t) \propto \left(\frac{\partial n}{\partial c} \right)^2 \frac{1}{\nu D} \frac{1}{1 + \left(\frac{q_{ro}}{q} \right)^4} \frac{(\nabla c)^2}{q^4} \quad (1)$$

where ν is the kinematic viscosity and D is the mass diffusion coefficient. Equation 1 describes the fast q^{-4} divergence at large q that is characteristic of non-equilibrium fluctuations that originate from the coupling between velocity fluctuations and concentration fluctuations^{4–6,15–20}. This is the same mechanism that gives rise to the large non-equilibrium fluctuations described in ref. 12. Equation 1 also describes the saturation at low q , where, as we will discuss later, any dependence on non-trivial fluid parameters actually cancels out. The transition between the two asymptotic behaviours occurs at the roll-off wavevector q_{ro} given by

$$q_{ro} = \left(\frac{\beta g \nabla c}{\nu D} \right)^{1/4} \quad (2)$$

where $\beta = \rho^{-1}(\partial \rho / \partial c)_{p,T}$, where ρ is the mass density and g is the gravitational acceleration.

The continuous lines through the data in Fig. 2 have been fitted according to equation 1 by floating the roll-off position and the asymptotic $I(q \approx 0)$ value. Best fitting of the asymptotic behaviour at large q was obtained by slightly changing the power-law dependencies in equation 1 (an exponent of 3.7 was used instead of 4).

The scattered intensity at small q , $I(q \approx 0)$, remains constant at first, but then it drops steadily. This behaviour is shown in detail in Fig. 3. If roughly the scattered intensity from the various layers of fluid is integrated (see equation 1), the overall scattered intensity at $q = 0$ becomes

$$I(q = 0) \propto \left(\frac{\partial n}{\partial c} \right)^2 \frac{\Delta c}{\beta g} \quad (3)$$

where Δc is the difference between the concentrations of the uppermost and lowermost layers of the sample. During the early

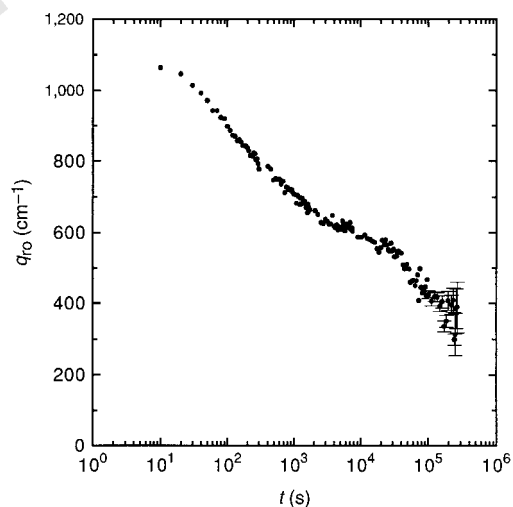


Figure 4 Roll-off wavevector q_{ro} as a function of time. The wavevector slowly decreases with time, indicating that fluctuations inside the sample grow larger as time goes by.

phases the diffusion proceeds mostly around the midplane, and Δc is constant (free diffusion regime). Later on the limitations of a restricted geometry are felt, and Δc diminishes steadily²¹, producing the behaviour shown in Fig. 3. The time at which the break in Fig. 3 occurs can be used to derive an estimate of the diffusion coefficient $D = (1.5 \pm 0.3) \times 10^{-6} \text{ cm}^2 \text{ s}^{-1}$. An estimate for D from reference data^{22,23} along the critical isotherm at $\delta c \approx 0.5$ gives $D = 1.8 \times 10^{-6} \text{ cm}^2 \text{ s}^{-1}$. Incidentally, the time constant τ associated with the fluctuations of the shadowgraph images (1 second) compares nicely with the estimate $\tau = 1/(Dq_{ro}^2)$. As $\Delta c = \delta c = 0.5$ and $\beta = 0.27$, we are in a position to calculate the theoretical value of the non-equilibrium forward-scattered intensity, relative to the equilibrium value. The value is shown in Fig. 2 by an arrow, and the agreement with the experimental data is within 40%, good agreement considering the uncertainty in reference data for $(\delta c / \delta \mu)$.

We emphasize that equation 3 shows that the low- q fluctuation amplitude does not depend on any relevant fluid parameter. So the orders-of-magnitude increase of the fluctuations above the equilibrium value (the most prominent feature that can be captured experimentally) is to be expected for any non-equilibrium fluid that has macroscopic concentration variations comparable to those in this experiment.

The roll-off position does depend on the transport coefficients ν and D . The time dependence of q_{ro} is shown in Fig. 4. It is known that during free, unbounded diffusion the concentration gradient at the midheight scales with the inverse square root of time³. From equation 2 then it follows that $q_{ro} = q_1 t^{-1/8}$, where the prefactor can be estimated from reference data (using our own determination $D = 1.5 \times 10^{-6} \text{ cm}^2 \text{ s}^{-1}$) to be $q_1 = 1.1 \times 10^3 \text{ cm}^{-1} \text{ s}^{1/8}$. The best fit of the data in Fig. 4 yields an exponent -0.11 ± 0.01 and a prefactor $q_1 = (1.5 \pm 0.06) \times 10^3 \text{ cm}^{-1} \text{ s}^{1/8}$. The agreement is satisfactory. The value of q_{ro} determines the largest wavelength at which the effects of the gravity are not felt^{12,16}. For lower q vectors the strong q^{-4} divergence is cut off. We must stress that the roll-off depends on g , and by reducing it the strong divergence becomes operative at longer wavelengths. We point out that in space experiments where g is really small, very large effects should be expected.

Equation 1 predicts that in the q^{-4} regime the scattered intensity scales as $(\nu D)^{-1}$. Consequently the choice of a near-critical fluid is advantageous in the large- q regime. For ordinary liquids $D \approx 10^{-5} \text{ cm}^2 \text{ s}^{-1}$ and therefore a factor of roughly ten is gained.

Finally, we point out that this work has strong connections with the area of convective instabilities, with special reference to the two-component Rayleigh–Bénard instability, where a temperature gradient is applied to a fluid slab^{24,25}. There too velocity and concentration modes couple, and large fluctuations are generated before the onset of the instability. Clear evidence of large pretransitional fluctuations has been presented²⁶. Indeed, it has been shown^{15,16} that the physical mechanism that leads in our case to the divergence cutoff is the same (but with opposite effects) as that which determines the instability threshold in the Rayleigh–Bénard case. □

Received 2 April; accepted 25 September 1997.

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Acknowledgements. This work was partially supported by ASI (Agenzia Spaziale Italiana).

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A manganese oxyiodide cathode for rechargeable lithium batteries

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The increasing demand for portable electronic devices is driving the development of compact lightweight batteries of high energy density¹. Lithium-ion batteries tend to be the systems of choice, as they offer higher energy densities and longer operational lifetimes than other rechargeable battery systems^{1,2}. But commercially available lithium-ion batteries make use of layered LiCoO_2 cathodes^{3,4}, and the high cost and toxicity of cobalt therefore motivate the development of cheaper and environmentally benign cathode materials. In this regard, manganese oxides are attractive alternatives, and the spinel LiMn_2O_4 has been investigated intensively as a cathode^{5,6}; however, the fading on cycling of its energy-storage capacity poses problems. More recently, attention has been focused on the synthesis of layered LiMnO_2 as a cathode material, but its cycling characteristics remain to be established^{7–9}. Here we report the synthesis and electrochemical performance of a new manganese oxide cathode, the oxyiodide $\text{Li}_{1.5}\text{Na}_{0.5}\text{MnO}_{2.85}\text{I}_{0.12}$. Our material exhibits a high reversible capacity of 260 mA h g^{-1} in the range 1.5–4.3 V with excellent cycling characteristics. Furthermore, the amorphous nature of the material (as determined by X-ray diffraction) and smooth discharge behaviour may help to overcome the problems associated with lattice distortions that have plagued manganese oxides with more crystalline structures^{5–9}.

Complex metal oxides are traditionally made by repeated grinding and firing of the raw materials at high temperatures in order to overcome the diffusional limitations. Such a 'brute force' high-temperature approach often leads not only to larger grain size and lower surface area, but also to an inaccessibility of metastable phases that may have unusual valences or atomic arrangements. This

awareness has created tremendous interest in designing low-temperature routes to synthesize complex materials¹⁰. We have shown¹¹ that alkali metal borohydrides such as NaBH_4 can be used effectively to reduce metalate ions $(\text{MO}_4)^{n-}$ (where M is V, Mo or W) in aqueous solutions to obtain binary oxides M_2O_3 and ternary oxides $\text{Na}_x\text{M}_y\text{O}_z$. The method gave amorphous or nanocrystalline phases, which were often metastable, and the binary oxides such as VO_2 and MoO_2 obtained by this approach were found to be attractive as electrode materials for lithium batteries^{12,13}.

Here we use LiI as a reducing agent to reduce the permanganate ion $(\text{MnO}_4)^-$ in acetonitrile medium. The use of a non-aqueous medium is particularly important to obtain electrode materials free from water. $\text{NaMnO}_4 \cdot \text{H}_2\text{O}$ was heated at 170°C for 2 hours to obtain anhydrous NaMnO_4 . A 0.05-M NaMnO_4 solution was then prepared by dissolving the anhydrous NaMnO_4 in acetonitrile. Anhydrous LiI was then added under constant stirring to the NaMnO_4 solution so that the molar ratio of NaMnO_4 to LiI is 1:1.5. After the mixture had been kept under constant stirring for about 1 day, the solid formed was filtered and washed several times with acetonitrile to remove the iodine formed during the reduction process.

The as-prepared sample was found to be amorphous to X-ray diffraction (Fig. 1a). The sample was then heated in vacuum at 250°C for about 10 h to remove the residual solvent acetonitrile or any adsorbed water before preparing the electrodes. The vacuum-annealed sample also did not show any sharp reflections (Fig. 1b), indicating that the sample was still amorphous to X-ray diffraction. However, after the sample had been annealed in air or N_2 atmosphere at a temperature $T > 300^\circ\text{C}$, reflections corresponding to Li_2MnO_3 and a small amount of $\text{Na}_{0.7}\text{MnO}_2$ were observed in the X-ray diffraction pattern (Fig. 1c). Figure 2 shows a micrograph recorded with a Jeol 200 CX transmission electron microscope (TEM), along with the selected area diffraction (SAD) pattern for the vacuum-annealed sample. The SAD pattern shows very diffuse rings, suggesting that the sample is not totally amorphous. It implies that short-range order may exist on the length scale of a few Mn–O polyhedra, which is not a long enough range to give well-defined diffraction patterns. This conclusion is also consistent with the development of a few broad reflections in the X-ray pattern of the vacuum-annealed sample (Fig. 1b) that are reminiscent of Li_2MnO_3 reflections.

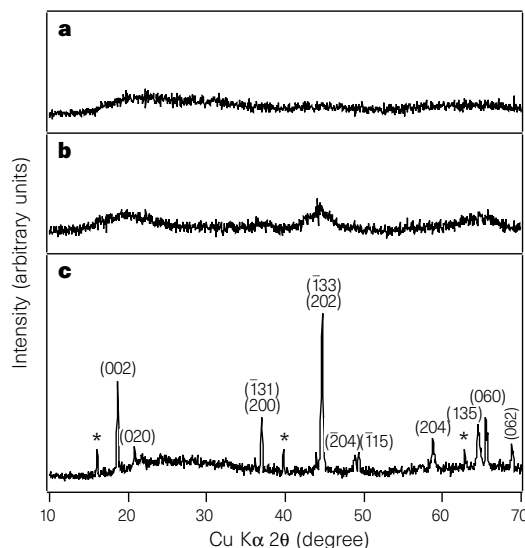


Figure 1 X-ray powder diffraction patterns of the product obtained by reducing permanganate with LiI. **a**, As prepared; **b**, after heating in vacuum at 250°C ; and **c**, after heating in air at 600°C . The planes marked in **c** refer to Li_2MnO_3 and the asterisk refers to $\text{Na}_{0.7}\text{MnO}_2$.

Energy-dispersive spectroscopic (EDS) analysis reveals the presence of Mn, Na and I in the as-prepared and vacuum-annealed samples. To fully characterize the composition, we determined the Li, Na and Mn contents by atomic absorption spectroscopy after dissolving the sample in dilute hydrochloric acid. The iodine content was determined by oxidizing the iodide to iodine with hydrogen peroxide in 1 M sulphuric acid solution¹⁴. The liberated iodine was then extracted with trichloromethane using a separating funnel and titrated with sodium thiosulphate. The oxidation state of Mn was determined by reducing the Mn^{n+} to Mn^{2+} with vanadyl sulphate in the presence of sulphuric acid and titrating the remaining vanadyl sulphate with potassium permanganate, as reported earlier¹⁵. The oxidation of I^- to I_2 during this redox process was taken into account in the calculation. The chemical analysis data reveal the vacuum-annealed sample to be $\text{Li}_{1.5}\text{Na}_{0.5}\text{MnO}_{2.85}\text{I}_{0.12}$. The chemical analysis data also indicate that the iodide content decreases with increasing firing temperature and the samples do not contain any iodide for firing temperatures $T \geq 600^\circ\text{C}$.

The vacuum-annealed sample with a composition of $\text{Li}_{1.5}\text{Na}_{0.5}\text{MnO}_{2.85}\text{I}_{0.12}$ was mixed with 25 wt% fine carbon and 5 wt% polytetrafluoroethylene (PTFE) to make electrodes. The electrochemical performance was evaluated with coin-type cells using a metallic Li anode and LiClO_4 in propylene carbonate/1,2-dimethoxyethane as the electrolyte. The first charge and the subsequent discharge–charge curves recorded with a current density of 0.1 mA cm^{-2} are shown in Fig. 3 for eight cycles. The first charge capacity of about 40 mA h g^{-1} corresponds to an extraction of about 0.2 Li per Mn. A maximum reversible capacity of about 260 mA h g^{-1} corresponding to an insertion/extraction of about 1.3 Li per Mn could be achieved in the range 4.3–1.5 V during the subsequent discharge–charge cycles with 100% capacity retention. The $\text{Li}_{1.5}\text{Na}_{0.5}\text{MnO}_{2.85}\text{I}_{0.12}$ electrodes thus show excellent reversibility involving both the $\text{Mn}^{4+/3+}$ and $\text{Mn}^{3+/2+}$ couples, unlike most other manganese oxide systems, which generally show reversibility only for the $\text{Mn}^{4+/3+}$ couple.

The discharge curves recorded with higher current density, and the cyclability, are shown in Fig. 4. Although the capacity is lower for higher current density, the material shows excellent cyclability without any fading of the capacity for at least the 40 cycles we have carried out so far. In fact, with higher current density, the capacity increases initially and tends to become stabilized at a higher value as the cell is cycled. Also, the discharge voltage increases slightly as the cell is cycled. We believe that the increase in capacity and discharge voltage is due to the relaxation of the nearly amorphous structure to create smooth pathways for the process of lithium insertion and extraction, thereby enhancing the lithium diffusion rate and lowering the cell resistance as the cell is cycled.

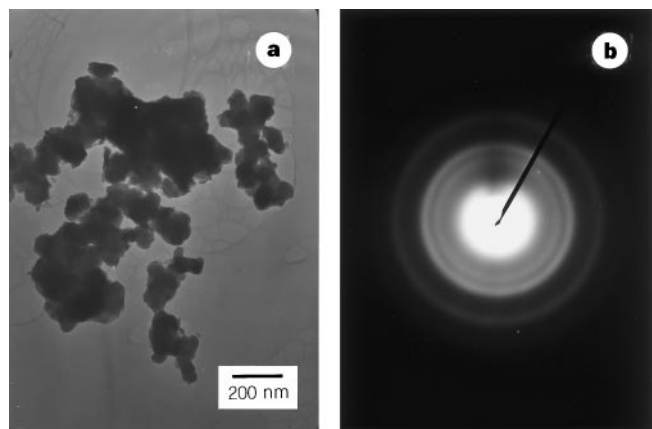


Figure 2 Vacuum-annealed $\text{Li}_{1.5}\text{Na}_{0.5}\text{MnO}_{2.85}\text{I}_{0.12}$. **a**, Transmission electron micrograph, and **b**, electron diffraction pattern.

Although at lower current density we have collected data for only eight cycles, the cyclability demonstrated for at least 40 cycles at higher current density indicates that the 260 mA h g^{-1} capacity could be delivered at lower current densities for large numbers of cycles without any fading.

The presence of a small amount of iodide not only keeps the material nearly amorphous but also may help, because of its larger size, to keep the space for lithium ions larger. The nearly amorphous nature leads to a larger capacity, as has been found with disordered carbon¹⁶. The amorphous nature may also be particularly useful in avoiding the common problems associated with the Jahn–Teller distortion of Mn^{3+} . The amorphous structure can smoothly accommodate the distortion without producing a macroscopic phase transition, unlike in the spinel LiMn_2O_4 . In addition, the smooth discharge curve with a uniform slope may provide the advantage of avoiding problems that may occur with over discharge or charge.

We have demonstrated that innovative low-temperature approaches can lead to potentially useful metastable electrode materials. Although $\text{Li}_{1.5}\text{Na}_{0.5}\text{MnO}_{2.85}\text{I}_{0.12}$ offers a lower mid-discharge voltage of 2.6 V, a larger capacity of 260 mA h g^{-1} leads to an energy density that is higher than those achieved practically with the layered LiCoO_2 (140 mA h g^{-1} at 3.7 V) or spinel LiMn_2O_4 (120 mA h g^{-1} at 3.8 V) cathodes¹⁷, and is comparable to that found

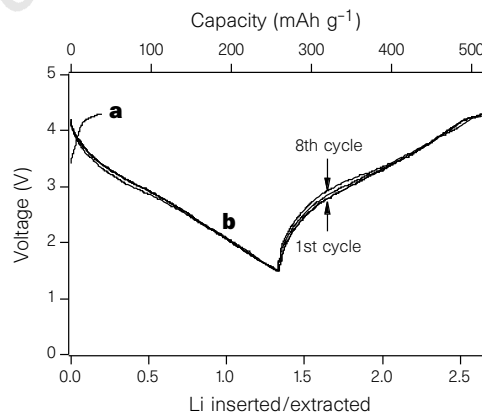


Figure 3 Charge and discharge–charge curves (8 cycles) of $\text{Li}_{1.5}\text{Na}_{0.5}\text{MnO}_{2.85}\text{I}_{0.12}$ recorded at a current density of 0.1 mA cm^{-2} . **a**, First charge, and **b**, subsequent discharge–charge curves.

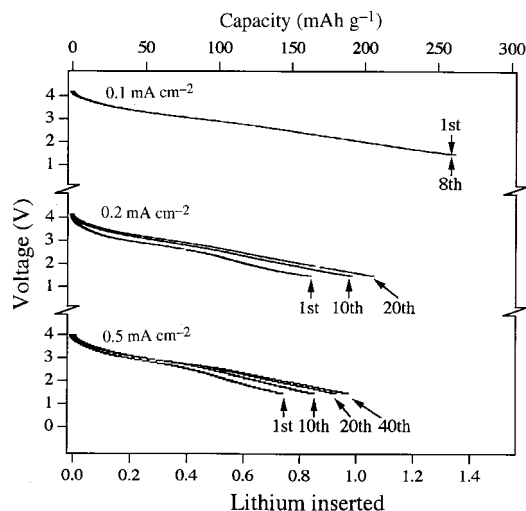


Figure 4 Discharge curves and cyclability of $\text{Li}_{1.5}\text{Na}_{0.5}\text{MnO}_{2.85}\text{I}_{0.12}$ recorded at various current densities.

for $\text{LiNi}_{0.85}\text{Co}_{0.15}\text{O}_2$ (180 mA h g^{-1} at 3.75 V)¹⁸. Although some MnO_2 samples show a high initial capacity, at lower current density comparable to the present oxydide, they show a marked decline in capacity after the first discharge and poor cycling ability¹⁹. The occurrence of more than 95% of the capacity below the 4 V range in the $\text{Li}_{1.5}\text{Na}_{0.5}\text{MnO}_{2.85}\text{I}_{0.12}$ cathodes may be particularly attractive for lithium polymer batteries as the polymer electrolytes may decompose above 4 V. In the future, the mechanism of lithium insertion into the amorphous structure will be investigated by solid-state nuclear magnetic resonance and X-ray absorption spectroscopies. Further research to optimize the cathode composition and microstructure, particularly to enhance the current density, is currently in progress. Initial chemical lithiation followed by annealing at $T < 250^\circ\text{C}$ may, for example, help to create a smooth pathway for lithium insertion and extraction, and to increase the initial capacity under higher current densities. □

Received 5 May; accepted 30 September 1997.

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Acknowledgements. This research was supported by the National Science Foundation.

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Implications of recent CO_2 emission-limitation proposals for stabilization of atmospheric concentrations

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The stabilization of atmospheric greenhouse-gas concentrations is the ultimate objective of the United Nations Framework Convention on Climate Change. To investigate the gas emissions required to achieve this goal for CO_2 over the next few hundred years, the Intergovernmental Panel on Climate Change (IPCC)^{1,2}

has used two sets of concentration pathways ('profiles'). One set is based solely on smooth changes in emission towards concentration stabilization³, and the other goes further by incorporating economic considerations⁴. Here I devise new profiles that take into account emissions-limitation proposals restricted to 'Annex I' (developed) countries, as well as the constraints imposed by more recent emissions data (now available until the end of 1995; ref. 5). The Annex I scenarios considered are two that span proposals recently considered by the IPCC⁶, and a less restrictive case. Using the new CO_2 -concentration profiles and a carbon-cycle model⁷ to determine global CO_2 emissions, and taking into account the prescribed Annex I country emissions, I determine the CO_2 -emission requirements for non-Annex I (developing) countries to achieve the stabilization of atmospheric concentration at about twice preindustrial values. I show that action by Annex I countries within the bounds of the scenarios considered can mean that non-Annex I countries have several decades before their emissions need to depart significantly from a 'business as usual' (no intervention) trajectory. Alternatively, if international trade in carbon emissions is permitted, non-Annex I countries can choose to emit below their 'business as usual' baseline and benefit from the trading of emission rights.

I devise new profiles for the stabilization of atmospheric CO_2 concentrations using the procedure used in ref. 4. CO_2 emissions are specified from 1990 to some future date (Y), concentrations (C) calculated to Y , and then a smooth profile fitted from $C(Y)$ to a range of stabilization concentrations. To specify the initial emissions trajectory over 1990 to Y , emissions for both Annex I and non-Annex I countries need to be defined.

For non-Annex I countries, I follow observed CO_2 emissions to 1995 (Table 1), project these linearly over 1995–2000, and then use changes as specified under the IPCC IS92a 'business as usual' (BAU) or 'no intervention' scenario (refs 6, 9, 10). For Annex I countries, I attempt to bracket the range of possibilities by considering three emissions-limitation scenarios (Fig. 2). All cases follow observed emissions through 1995 (Table 1). In two cases, emissions increase linearly from 1995 to a value in 2000 that is equal to the 1990

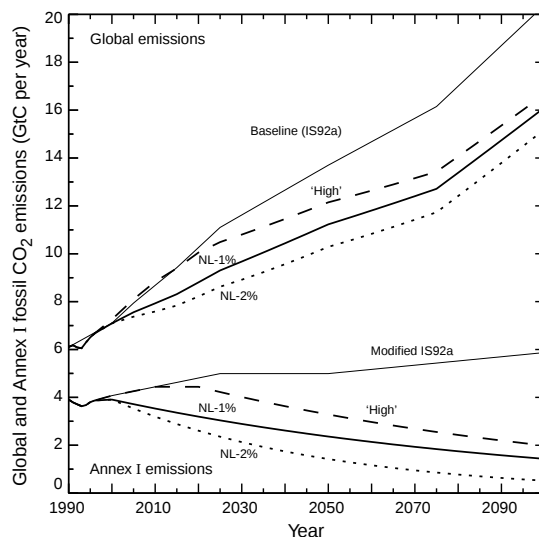


Figure 1 Global (upper curves) and Annex I country (lower curves) fossil CO_2 emissions (GtC per year) for the central IPCC 'existing policies' emissions scenario (IS92a)^{9,10} compared with cases where Annex I emissions are limited according to the NL-1%, NL-2% and 'High' scenarios described in the text. For global emissions, the IS92a values shown here are precisely those given by the IPCC. For Annex I emissions in the IS92a case, observed values are used through 1995, and changes from 1995 are as in the original IS92a scenario.

emissions level; returning to this level by 2000 is a common (but probably unrealistic) feature of many of the current emissions-limitation proposals⁶. From 2000, emissions decline at 1% or 2% (compound) per year. These cases are virtually identical to the NL-1% and NL-2% scenarios proposed by the Netherlands and analysed by the IPCC⁶. For the third case, changes in Annex I emissions over 1995–2010 follow the BAU scenario. Emissions remain constant over 2010–2020 and decline at 1% (compound) per year from 2020 onwards. I refer to this as the 'High' scenario. To account for bunker fuels, we use a constant value equal to that for 1995 (Table 1). The 'High' scenario is less stringent than NL-1% and NL-2%, and is less restrictive than most of the proposals recently put forward by Annex I countries. Global and Annex I emissions for these scenarios are shown in Fig. 1.

In terms of non-Annex I emissions, the scenarios represent 'unrestricted' cases where no emissions limitations are imposed. Concentration will not stabilize in any of these cases: for stabilization, global emissions must depart at some future date below these 'unrestricted' cases. I consider a range of different departure dates (years Y), 2010, 2030, 2050 and 2070. Because Annex I emissions are fully specified, these dates correspond to the points at which non-Annex I emissions depart from the unrestricted cases.

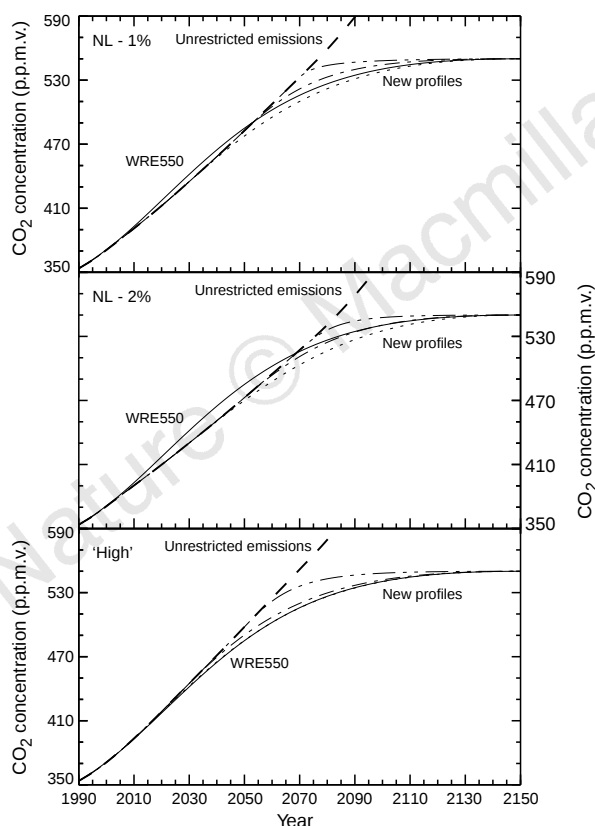


Figure 2 Revised CO₂-concentration stabilization profiles. Top, CO₂-concentration stabilization profile accounting for recent emissions data and the proposed NL-1% limitation to Annex I emissions (1% per year decline from the 2000 emissions level). The unrestricted-emissions concentration projection is shown as a thick, dashed line; the three stabilization cases (thin broken lines) are assumed to depart from this projection in 2030, 2050 and 2070, and reach a stable CO₂ concentration of 550 p.p.m.v. in 2150. The WRE550 stabilization profile⁴ is shown for comparison. Middle, as for top, but with the unrestricted emissions case following the NL-2% scenario for Annex I emissions (2% per year decline from the 2000 level). Bottom, as for top, but with the unrestricted-emissions case following the 'High' scenario for Annex I emissions.

Given the unrestricted global emissions, a carbon-cycle model⁷ is used to determine unrestricted concentrations. The concentration in the chosen departure year ($C(Y)$), together with the selected stabilization level, is then used to specify the stabilization profile (see refs 3, 4). Finally, the carbon-cycle model is used again, in inverse mode, to determine the emissions required to follow the profile; this necessarily recovers the input emissions up to year Y.

These calculations are subject to a number of uncertainties^{1–3}, albeit reduced somewhat by the constraint on emissions up to year Y. For the concentration profiles, uncertainties arise from uncertainties in $C(Y)$. These may be estimated by varying the 1980s-mean value of net land-use emissions (Dn80s) used in initializing the model (see refs 1–3, 7, 8). Using a range of 0.4–1.8 GtC per year for this quantity leads to $C(Y)$ uncertainties of ± 5 p.p.m.v. in 2010, ± 12 p.p.m.v. in 2030 and ± 20 p.p.m.v. in 2050.

For the inverse-calculated emissions the uncertainties are relatively small because, no matter what value of $C(Y)$ is used to define the concentration profile, the emissions are constrained to be correct to year Y. Beyond this date, emissions uncertainties will increase towards values that are determined largely by the behaviour of the terrestrial biosphere, in turn reflecting human-induced land-use changes, climate change, and possible changes in the magnitude

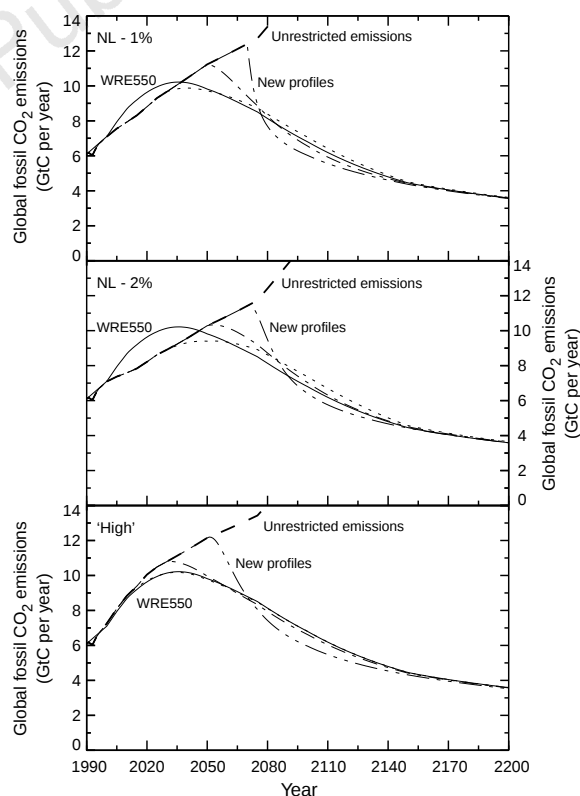


Figure 3 Global fossil CO₂ emissions. Top, global fossil CO₂ emissions based on the CO₂-concentration stabilization profiles shown in the top panel of Fig. 2, that is, assuming that Annex I countries limit their post-2000 emissions according to the NL-1% scenario. The unrestricted-emissions projection is shown as a thick, dashed line; the three stabilization cases are shown as thin, broken lines; and the WRE550 case is shown as a thin solid line. Middle, global fossil CO₂ emissions based on the CO₂-concentration stabilization profiles shown in the middle panel of Fig. 2, that is, assuming that Annex I countries limit their post-2000 emissions according to the NL-2% scenario. Bottom, global fossil CO₂ emissions based on the CO₂-concentration stabilization profiles shown in the bottom panel of Fig. 2, that is, assuming that Annex I countries limit their post-2010 emissions according to the 'High' scenario.

of the CO₂-fertilization sink; I do not consider these factors here. Such uncertainties could range from zero in year *Y* up to 1 GtC per year by 2100. However, these uncertainties have little influence when comparing results for different emissions-limitation cases (NL-1% versus NL-2% versus 'High') or for different departure dates.

For specificity, I examine the implications of Annex I emissions limitations for concentration stabilizing at 550 p.p.m.v. in the year 2150. This level is chosen because it is in the centre of the 350–750 p.p.m.v. range that has been the focus of IPCC studies^{1–3}. It would be of interest to carry out similar calculations for a range of stabilization levels, given that no target has yet been specified and that the economic costs and potential environmental damages depend critically on the chosen target².

I first consider the NL-1% emissions-limitation scenario. With no restriction on non-Annex I emissions, concentrations rise to 590 p.p.m.v. in 2090, passing 550 p.p.m.v. in 2077 (Fig. 2). Up to 2053, the NL-1% case has concentrations below the equivalent (WRE550) profile derived previously⁴ for the case where there are no Annex I restrictions. Thus, restricting the emissions of Annex I countries in this way is more than enough to progress towards stabilization at 550 p.p.m.v. at least to 2053. However, one cannot

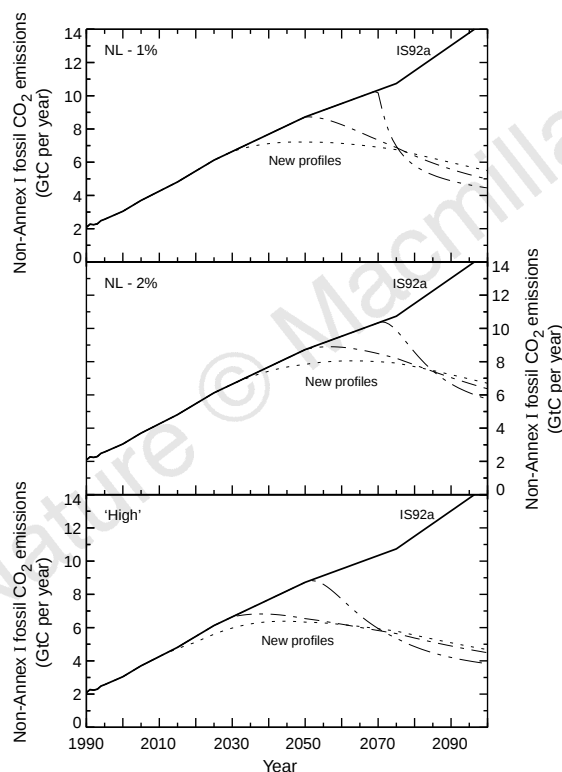


Figure 4 Fossil CO₂ emissions required for non-Annex I countries. Top, fossil CO₂ emissions required for non-Annex I countries if CO₂ concentration is to stabilize at 550 p.p.m.v., based on the global emissions results shown in the top panel of Fig. 3 and assuming that Annex I countries limit their post-2000 emissions according to the NL-1% scenario. The curve labelled 'IS92a' (bold line) is the unrestricted-emissions case, based on observed emissions to 1995, and IS92a changes subsequently. The broken curves ('New profiles') correspond to departure dates for IS92a of 2030, 2050 and 2070. Middle, fossil CO₂ emissions required for non-Annex I countries if CO₂ concentration is to stabilize at 550 p.p.m.v. based on the global emissions shown in the middle panel of Fig. 3 and assuming that Annex I countries limit their post-2000 emissions according to the NL-2% scenario. Bottom, fossil CO₂ emissions required for non-Annex I countries if CO₂ concentration is to stabilize at 550 p.p.m.v., based on the global emissions results shown in the bottom panel of Fig. 3 and that assuming Annex I countries limit their post-2010 emissions according to the 'High' scenario.

simply move from the NL-1% pathway to the WRE550 profile in 2053, because the implied discontinuity in the rate of change of concentration will lead to an unattainably rapid emissions change. We therefore consider three examples in which the concentration profile departs smoothly from the NL-1% case in 2030, 2050 and 2070 (Fig. 2).

Global fossil CO₂ emissions for the different profiles are shown in Fig. 3. The WRE550 case is shown for comparison. To the extent that WRE550 is economically attainable, so too is the stabilization pathway that deviates from the unrestricted pathway in 2030; the two show very similar emissions and rates of change of emissions. WRE550, by definition, departed from the IS92a 'no-intervention' emissions pathway in 2010, implying that the NL-1% limitation scenario for Annex I countries has 'gained' non-Annex I countries an additional 20 years before their CO₂ emissions must deviate significantly from the BAU course. (This does not, of course, mean that non-Annex I countries could or should delay action until 2030.) The later departure dates of 2050 and 2070 would be harder to achieve as they require a much more rapid transition from increasing to decreasing fossil CO₂ emissions (Fig. 3).

Figure 4 shows the emissions for non-Annex I countries, obtained by subtracting the bunker fuel and Annex I emissions under NL-1% from the inverse-calculated global emissions shown in Fig. 3. Part of the decline in the three global emissions pathways is taken up by the decline of 1% per year in Annex I emissions, leaving a slower decline for non-Annex I countries. The 2050 and 2070 departure-date cases still require very rapid transitions from increasing to decreasing emissions, but the 2030 departure case has a long period of near-stable emissions.

If Annex I countries were to reduce emissions more rapidly than in the NL-1% case, this would further reduce the need for early departures below BAU emissions for non-Annex I countries. To quantify this, I consider the NL-2% case. Results analogous to the NL-1% case are shown in the middle panels of Fig 2–4. The additional Annex I emissions reductions must, of course, lead to smaller and/or later restrictions for non-Annex I countries. For departure from the unrestricted case in 2030, their implied emissions rise higher and maximize at a later date than NL-1%. For a departure date of 2050, emissions reductions below the unrestricted case are significantly less than for NL-1%, and the required emissions decline is noticeably less abrupt. Emissions declines for the 2070 departure date are also slower than in the NL-1% case, but still appear to be unattainably rapid.

NL-2% corresponds to larger restrictions on Annex I emissions compared with NL-1%. The 'High' case (Fig. 1) corresponds to smaller and less rapid restriction; indeed, no restrictions at all until 2010. (It should be noted, however, that the reductions relative to

Table 1 Fossil CO₂ emissions breakdown

Year	Annex I* (GtC per year)	non-Annex I (GtC per year)	Bunker fuels† (GtC per year)	Total (GtC per year)
1990	3.907	2.088	0.114	6.109
1991	3.791	2.270	0.117	6.178
1992	3.708	2.240	0.136	6.084
1993	3.631	2.293	0.129	6.053
1994	3.676	2.484	0.132	6.292
1995	3.801	2.567	0.138	6.506
1996	3.869‡	2.662§	0.138	6.669¶

The data in this table are taken from ref. 5, updated by G. Marland (personal communication). Fossil CO₂ emissions given are from fossil-fuel combustion (including gas flaring), cement production, and other non-fuel carbon sources.

* Annex I countries are: Australia, Austria, Belarus, Belgium, Bulgaria, Canada, Czechoslovakia, Denmark, European Economic Community, Estonia, Finland, France, Germany, Greece, Hungary, Iceland, Ireland, Italy, Japan, Latvia, Lithuania, Luxembourg, Netherlands, New Zealand, Norway, Poland, Portugal, Romania, Russian Federation, Spain, Sweden, Switzerland, Turkey, Ukraine, United Kingdom, and United States of America.

† Bunker fuels are those used in air and marine international transport.

‡ By difference from assumed values for total, non-Annex I and bunker-fuels emissions.

§ Linearly extrapolated from 1995 to 1990–1995 rate.

|| Assumed equal to the 1995 value.

¶ Based on an estimated increase of 2.5% over the 1995 value.

BAU after 2010 in this scenario could only be achieved if decisions were made now with regard to future energy production, so this case certainly does not correspond to a 'no action' scenario.) Results for the 'High' case are shown in the bottom panels of Fig. 2 (concentration profiles based on non-Annex I departure dates of 2010, 2030 and 2050), Fig. 3 (implied global fossil CO₂ emissions) and Fig. 4 (implied emissions for non-Annex I countries).

For the 2010 departure date, the concentration profile is indistinguishable from WRE550, and the implied global emissions are virtually the same. This is not surprising, as the 'High' scenario assumes that emissions begin to decline below the IS92a 'no-intervention' case in 2010 for both groups of countries. This is the same as the assumption on which WRE550 was based, but here I specify the way global emissions reductions are apportioned between Annex I and non-Annex I countries. The departure dates of 2030 and 2050 correspond to different ways of apportioning the emissions restrictions: 2030 allows larger emissions for non-Annex I countries but requires a slightly earlier maximum and a slightly more rapid decline, whereas 2050 allows a much larger emissions maximum for non-Annex I countries but requires a very rapid decline.

In this analysis I have assumed that there is no coupling between emissions in Annex I and non-Annex I countries; such coupling could lead either to greater emissions in the latter group ('carbon leakage' through energy price or trade effects), or lower emissions (through technology diffusion or transfer). Coupling would also occur if there were trading of emissions rights between countries in the two groups. My results can easily be generalized to cover this possibility.

If A represents Annex I emissions, N represents non-Annex I emissions, and T is the emissions traded, then the present results may be considered as applying to 'effective' Annex I and non-Annex I emissions, defined by $A - T$ and $N + T$, respectively. The scenarios in Fig. 2 would then represent scenarios for $A - T$; actual Annex I emissions (A) would exceed these by the amount traded. Non-Annex I emissions would be less than the BAU values assumed here (by an amount T), taking advantage of economic efficiencies and/or technological advances that might diffuse from Annex I countries. The pathways shown here may then be considered as spanning a range of trading (or burden sharing) possibilities. Economic analyses could then be used to assess the relative costs of the different Annex I emissions-limitation cases, and, within these, the relative costs of different departure dates for non-Annex I emissions from BAU and of a range of assumptions with regard to the trading of emissions rights.

In summary, I have shown how the emissions-limitation burden to achieve CO₂ stabilization at 550 p.p.m.v. (roughly double the preindustrial level) might be shared between Annex I and non-Annex I countries. Choosing between different possibilities and between different stabilization levels requires careful analysis of their environmental and economic consequences. Reassuringly, even if Annex I emissions were to stay close to BAU until 2010 (as in the WRE550 case), this would not preclude stabilization. If, in this case, Annex I emissions were then to decline relative to BAU at the rates assumed here, this would allow emissions in non-Annex I countries to follow close to BAU until around 2030. More stringent (or earlier) Annex I restrictions would allow a later departure from BAU for non-Annex I countries. The result that significant departures below BAU emissions in non-Annex I countries could occur decades after those assumed for Annex I countries, when combined with the possibility of substantial wealth transfers through emissions trading from Annex I to non-Annex I countries, provides a foundation for a fair and equitable solution to the problem of climate change. □

Received 15 September; accepted 31 October 1997.

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Acknowledgements. I thank G. Marland for pre-publication access to the emissions data given in Table 1, and R. Richels for suggestions on improving an earlier version of this paper. This work was supported by the US Department of Energy. NCAR is sponsored by the National Science Foundation.

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Influence of socioeconomic inertia and uncertainty on optimal CO₂-emission abatement

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Following the United Nations Framework Convention on Climate Change¹, governments will negotiate, in Kyoto this December, an agreement to mitigate anthropogenic greenhouse-gas emissions. Here we use a model approach to examine optimal CO₂-emission abatement paths for specified long-term constraints on atmospheric CO₂ concentrations. Our analysis highlights the interplay of uncertainty (in target greenhouse-gas concentrations) and the inertia in the energy systems that produce CO₂ emissions. We find that the 'integrated assessment' models previously applied to these issues under-represent inertia. A more appropriate representation of inertia increases the costs of deferring abatement and makes it optimal to spread the effort of abatement across generations. Balancing the costs of early action against the potentially higher costs of more rapid and forced later action, we show that early attention to the carbon-emitting potential of new and replacement energy investments will minimize the risk to environmental and economic systems. We conclude that if there is a significant probability of having to maintain atmospheric greenhouse gas concentrations below about double those of the pre-industrial era, then the economic risks associated with deferring abatement justify starting to limit CO₂ emissions from energy systems immediately.

Policy towards climate change faces uncertainty about the ultimate goal: we are not likely to know soon at what atmospheric concentration of greenhouse gases "dangerous interference with the climate system"¹ would occur. An initial emissions-abatement path consistent with reaching one selected concentration ceiling may have to be either accelerated or relaxed in the light of new scientific

information. This is why the IPCC states that: "The choice of abatement paths involves balancing the economic risks of rapid abatement now (that premature capital stock retirement will later be proven unnecessary), against the corresponding risks of delay (that more rapid reduction will then be required, necessitating premature retirement of future capital)"².

Incorporating such uncertainty into the analysis implies that the inertia of the economic systems producing greenhouse gases becomes critical. Energy production systems cannot be changed overnight and energy demand is driven by long-lasting patterns of infrastructure and behaviour. Without inertia, the transition costs for switching from one emission path to another would be null, and uncertainty would be less critical. But in a stochastic framework, inertia raises both the costs of premature abatement and the costs of accelerating abatement if stronger action is called for after a period of delay.

Here we analyse these issues using a compact intertemporal optimization model, DIAM, defined in Box 1. DIAM determines the least-cost CO₂-emission pathway consistent with staying below a given or stochastic atmospheric CO₂-emission pathway consistent with staying below a given or stochastic atmospheric CO₂ concentration ceiling, given assumptions concerning abatement costs; the rate r at which these costs are reduced by exogenous technical progress (that is, technical advance that is assumed to be independent of emissions abatement); the societal discount rate ρ (the annual rate of decline in the present value of costs and benefits incurred in the future); and the inertia in the system, D . We adopt parameters lying within the range of magnitude quoted in the literature for exogenous technical progress (1% per year) and for the societal discount rate (3% and 5% per year)¹², and for the model of carbon accumulation (Box 1). The inertia parameter, D , derived from the structure of abatement costs, is the novel aspect of the model and demands more discussion.

The abatement cost at time t is expressed as a quadratic function of both the degree and the rate of abatement (Box 1, equation 6). The nonlinearity of costs with respect to the degree of abatement x is well established². By adding a term that depends upon the rate of abatement, A (akin to the time derivative of x), the model captures inertia explicitly. Modelling studies of capital stock turnover³ confirm that costs depend in a nonlinear manner on the rate of abatement. In equation 6, additive separability between permanent costs (in $c_a(D)x^2$) and adjustment costs (in $c_a(D)D^2A^2$) makes it possible to explore the impact of transitional costs independently of long-run, permanent abatement costs⁴. For example, various transportation systems and urban planning patterns with very different carbon-emission potentials could have comparable operation costs. Innovations induced by carbon emissions constraints and cumulating over long periods could also reduce long-run costs to low levels. Nevertheless, starting one policy and then switching to another may entail high transition costs.

To test the sensitivity of our results with respect to the form of the cost function, we explore an alternative-specification equation 6a, in which adjustment costs are determined solely by comparing A with the rate of capital depreciation in the energy sector. As long as A is lower than the 'natural' pace of replacement of capital $1/D$, there are no adjustment costs and the reduction cost is proportional to x^2 ; above that pace, costs would be multiplied by $\max(1, DA)$.

Dimensional analysis in both specifications shows that D is a duration which can be interpreted as the characteristic timescale of changes in the global energy system. If interpreted purely in terms of capital depreciation at rate δ , then $D = (\ln 2)/\delta$.

This approach allows us to capture crucial dynamic constraints without resorting either to the arbitrary upper bound on emission reduction rates (as in refs 5, 6, 7) or to the limitations associated with representing inertia purely in terms of uniformly depreciating capital stock (as in ref. 3, 8, 9). DIAM gives approximately the same results as these latter models when we set $D = 20$ years (that is

$\delta \approx 4\%$ per year), a reasonable average for the rate of renewal of appliances, cars, power stations or refineries, the lifetimes of which range from 10 to 40 years.

But there are many other sources of inertia in socioeconomic systems that produce greenhouse gases. Time is needed to remove market and institutional barriers to the diffusion of innovations, and obstacles arising from imperfect information and imperfect foresight. As has been demonstrated empirically¹⁰, without specific policies, new energy sources have taken about 50 years to penetrate from 1% to only 50% of their ultimate potential. Furthermore, anthropogenic greenhouse-gas emissions depend in part on long-lived capital, such as buildings, transport and urban infrastructures with effects that may last more than 50 years, and on the linkages between different systems, such as the complex interlinked investments in mines, ports and power stations. Greenhouse-gas emissions today are still clearly influenced, perhaps strongly, by planning

Box 1 DIAM: a model of the dynamics of inertia and adaptability for integrated assessment of climate-change mitigation

Indices t, u refers to time periods; s refers to states of the world. DIAM⁴ finds the optimal abatement strategy $x^*(s, t)$ by minimizing the total expected discounted abatement cost J , defined in equation (1) under constraints in equation (2), the concentration ceiling, and equation (3), dynamic programming. Equation (4) defines CO₂ atmospheric concentration $M(s, t)$, and equation (5) defines the acceleration of abatement $A(s, t)$. Abatement costs $C(s, t)$ are defined by equation (6). Alternatively, equation (6a) is used to test the sensitivity of the results to the shape of the cost function.

Time profile $E^{\text{ref}}(t)$ refers to anthropogenic fossil carbon emissions in IPCC scenario IS92a. The reference CO₂ concentration path $M^{\text{ref}}(t)$ is computed from IS92a total carbon emission with the atmospheric perturbation CO₂ response function $R(u)$ using a linear carbon cycle^{6,12} (model W). $M^{\text{ref}}(1765)$, the CO₂ concentration before industrialization, was less than 280 p.p.m.v. The CO₂ concentration was 354 p.p.m.v. in 1990, increasing at 1.7 p.p.m.v. per year (ref. 6).

Reduction costs are determined by a social risk-free discount rate ρ (3% or 5%, to account for pending controversies); a technical progress rate r (1%), and a characteristic time of energy systems D (20 or 50 years). The costs scale $c_a(D)$ is normalized ($c_a(50) = 1.36$, $c_a(20) = 3.18$) so that total cost is similar to DICE's cost⁴. Note that when equation (6) is reported into equation (1), the $c_a(D)$ need no longer be under the integral, implying that the optimal $x^*(s, t)$ is independent of $c_a(D)$.

The stochastic concentration ceiling is defined by the number of alternatives examined, N ; ceiling levels $L(s)$; subjective probabilities $p(s)$; and the date of uncertainty resolution t_{info} .

Results are shown in Table 1. First we examine certainty scenarios $N = 1$ for different values of D , ρ and L (sensitivity to r is mathematically the same as sensitivity to ρ). Then we explore $N = 3$ with equidistribution over {450, 550, 650} p.p.m.v., for $t_{\text{info}} = 2020$ and $t_{\text{info}} = 2035$.

$$J = \sum_{s=1}^N p(s) \sum_{t=1987}^{t=2000} (1 + \rho)^{t-1} C(s, t) \quad (1)$$

$$M(s, t) \leq L(s) \quad (2)$$

$$\forall t \leq t_{\text{info}}, \forall s, 1 \leq s \leq N, x(s, t) = x(1, t) \quad (3)$$

$$M(s, t) = M^{\text{ref}}(t) - 0.471 \sum_{u=t_0}^{u=t-1} R(t-u) x(s, u) E^{\text{ref}}(u) \quad (4)$$

$$A(s, t) = x(s, t) - x(s, t-1) \quad (5)$$

$$C(s, t) = c_a(D)(1+r)^{t_0-t} \frac{E^{\text{ref}}(t)}{E^{\text{ref}}(t_0)} [x(s, t)^2 + D^2 A(s, t)^2] \quad (6)$$

$$C(s, t) = c_a(D)(1+r)^{t_0-t} \frac{E^{\text{ref}}(t)}{E^{\text{ref}}(t_0)} [x(s, t)^2 \max(1, DA(s, t))] \quad (6a)$$

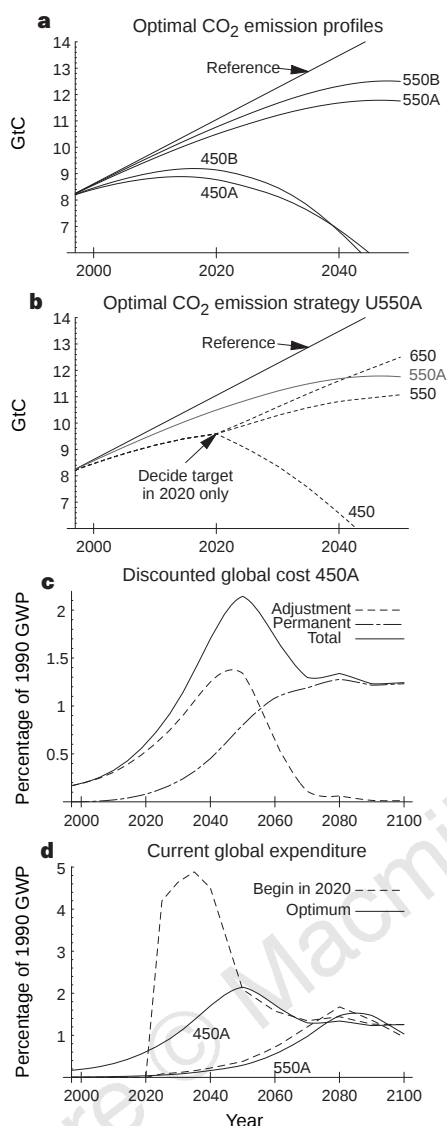


Figure 1 Optimal pathways under a given stabilization constant. The reference case, emissions increasing linearly by about 2% per year from the 1997 level, approximates the IPCC IS92a emissions scenario¹⁴. This is assumed to be least cost without constraints, so abatement shown is global average over and above any 'no regret' reductions. One GtC equals $[12 + (2 \times 16)]/12 = 3.67 \times 10^{12}$ kg of CO₂. **a**, Four optimal emission pathways that minimize the total abatement costs for the given concentration ceiling, inertia and discount rate (see Table 1). The B cases have lower inertia and higher discount rate than A, both factors leading to higher near-term optimal emissions. However, optimal policy is more sensitive to the ultimate concentration ceiling, 450 or 550 p.p.m.v. **b**, Optimal emission strategy U550A under stochastic constraint. The ultimate target is decided only in 2020, before which the strategy follows a precautionary path emitting less than the comparable case without uncertainty 550A. **c**, Cost as the sum of adjustment cost and permanent cost, optimal path 450A. The ratio: area under the 'Adjustment' curve discounted divided by area under the 'Total' curve discounted is the share of adjustment costs, labelled Adj./total in Table 1. **d**, Current expenditure profiles with (broken lines) and without (solid lines) a 20-years delay, for 450 and 550 p.p.m. stabilization targets (case A). For 450 p.p.m., cost peaks abruptly and is much higher with delay than without, owing to higher adjustment costs in the 2020–2040 period. Results are not that sharp for 550 p.p.m., as the time available to stabilize at 550 p.p.m. (or above) exceeds the characteristic time of the global energy system. In **c** and **d** the vertical axis unit is percentage of 1990 gross world product (GWP), assuming the Nordhaus cost function⁷. Because $D = 50$ for all curves in **b**, **c** and **d**, uncertainties in the cost scale parameter $c_a(50)$ would only affect the curves in equal proportion.

and investment decisions made in the decade after the Second World War.

None of these additional sources of inertia are reflected in available 'integrated assessment' models. Such models do not explicitly represent energy-consuming infrastructure such as buildings or roads, or the linkages between different parts of supply systems. Nor do they capture issues of technology clustering (for example, the complex set of interlinked vehicle, fuel refining and delivery technologies required for modern transport) or adaptive responses associated with induced innovation, which cumulate over decades. Other critical aspects, such as the dynamics of population, economic growth and induced technology development and diffusion, within and beyond the energy sector itself, may also involve very long timescales, and these too are not captured in the current 'integrated assessment' models¹⁵.

This difference between inertia in current modelling practice (~20 years) and the larger empirical value (~40–60 years) is therefore not surprising. Consequently we explore both 20 years and 50 years for the values of D ; with these values, adjustment costs represent from 18% to 71% of the total cost, depending upon the situation (Table 1, Fig. 1c).

We focus initially on atmospheric concentration ceilings of 450 and 550 p.p.m.. CO₂ concentrations in the range 450–500 p.p.m., depending on assumptions about other greenhouse gases, correspond to a total radiative forcing about double the preindustrial

CO₂ concentration, which has been the benchmark for most climate model analyses of future equilibrium climate change¹¹. The higher value of 550 p.p.m. represents the level given greatest attention in ref. 12. Under the IPCC's IS92a emissions scenario (ref. 14) 450 p.p.m. will be passed around the year 2030, and our optimal scenarios converge to 450 p.p.m. as a ceiling in 2050–2060; 550 p.p.m. would be passed at around 2065 and converges as a ceiling in 2080–2100. Corresponding optimal pathways under different assumptions are illustrated in Fig. 1a. A value of 650 p.p.m. is not reached until the end of the century or even later.

Our main results regard the interaction of inertia and uncertainty for trajectories in which abatement can start from 1997. The top of Table 1 represents optimal trajectories to a concentration ceiling known from the start, whereas the bottom represents optimal strategies when 550 p.p.m. is the expected value of three equiprobable ultimate CO₂ concentration limits of 450, 550 or 650 p.p.m. (Fig. 1b). For deterministic ceilings, the optimal trajectory is most sensitive to the ceiling. Optimal global abatement in 2020 (over and above any 'no-regret' measures) ranges from 19% to 24% for the 450 p.p.m. ceiling, and 3% to 7% for the 550 p.p.m. case. When 550 p.p.m. is the average of a stochastic target, however, optimal abatement is 9–14%. Furthermore, the results are now quite sensitive to the assumed inertia. With $D = 50$ years, optimal global abatement in 2020 is 11–14%; only with low inertia and high discounting does it drop below 10%.

Table 1 Characteristics of optimal global emissions scenarios and strategies

Certainty scenario	<i>D</i> (years)	ρ	<i>L</i> (p.p.m.v.)	<i>t</i> _{stab}	<i>X</i> ₂₀₂₀	<i>X</i> ₂₀₂₀ (equation 6a)	<i>E</i> _{max} (GtC)	<i>t</i> _{<i>E</i>max}	Adj./total	Cost of delay
450A (Fig. 1a, c, d)	50	3%	450	2060	24%	18%	8.7	2015	59%	+70%
450B (Fig. 1a)	20	5%	450	2050	19%	14%	9.2	2015	32%	+32%
450C	50	5%	450	2050	20%	16%	9.1	2015	74%	+72%
450D	20	3%	450	2060	23%	19%	8.8	2015	19%	+25%
550A (Fig. 1a, b, c)	50	3%	550	2100	7%	4%	11.5	2050	55%	+10%
550B (Fig. 1a)	20	5%	550	2080	3%	2%	12.6	2050	31%	+2%
550C	50	5%	550	2090	4%	2%	12.2	2050	71%	+8%
550D	20	3%	550	2090	5%	4%	11.9	2050	18%	+3%
650A	50	5%	650	2125	3%	0%	14.1	2070	55%	+4%
Hedging strategy	<i>D</i> (years)	ρ	<i>N</i>	<i>t</i> _{info}	<i>X</i> ₀₂₀	<i>X</i> ₂₀₂₀ (equation 6a)	<i>X</i> ₂₀₁₀	<i>E</i> ₂₀₂₀ GtC		
U550A (Fig. 1b)	50	3%	3	2020	14%	15%	8%	9.6		
U550B	20	5%	3	2020	9%	6%	4%	10.1		
U550C	50	5%	3	2020	11%	12%	6%	9.9		
U550D	20	3%	3	2020	12%	9%	7%	9.8		
U550L _(late)	50	3%	3	2035	21%	17%	12%	8.9		

Reduction figures refer to the global average (initial reductions focused on developed countries would be proportionately greater), and to reductions additional to those not incurring significant economic costs (that is, reductions beyond 'no regret' measures). Column *t*_{stab} shows the optimal stabilization date; *X*₂₀₂₀ and *X*₂₀₁₀ are the industrial-emissions abatement in 2020; (*E*_{max}, *t*_{*E*max}) is the optimal emissions curve apex; Adj./total is the share of adjustment costs in total costs (see Fig. 1c, legend); the cost of delay is the increase in discounted total cost when reduction starts in 2020 instead of 1997; and *E*₂₀₂₀ is the optimal emissions level in 2020.

The explanation for this result is to be found in Table 1 and Fig. 1c, d. Under a deterministic 450-p.p.m. ceiling, the main early expenditures arise from the transitional costs of turning the system away from the reference trajectory; for *D* = 50 these amount up to 59% of the total discounted costs. Deferring abatement requires this effort to be squeezed into a much shorter period of time, as also demonstrated in ref. 13. Under the 450 p.p.m. ceiling, deferring abatement by two decades adds 70% to the total cost for *D* = 50, but only 32% for *D* = 20. As illustrated by the results for 550 p.p.m., the costs of deferring abatement is not nearly as large when the time to stabilization is much greater than the characteristic time of the socioeconomic system. Consequently, when the ultimate target is uncertain, the costs of switching to the 450-p.p.m. pathway too late far exceeds the costs of premature abatement in the 650-p.p.m. case. This effect is all the more important if the resolution of uncertainties comes later (scenario U550L).

Even with low inertia and high discounting, corresponding to most of the models cited in ref. 12, the abatement under uncertainty (U550B) is 9% in 2020 compared with 3% for the deterministic equivalent. But recognizing higher inertia and the extent of uncertainties amplifies the economic risks associated with deferring abatement.

The results of Wigley, Richels and Edmonds¹², drawing on various integrated assessment models of climate change such as DICE (ref. 8) or MERGE2 (ref. 9), have been widely interpreted to support a policy of modest early abatement. Our analysis, like theirs, neglects the role of early abatement in stimulating learning-by-doing (which would reduce subsequent abatement costs⁴) and in deferring and slowing the direct impacts of climate change itself. Even neglecting these benefits, our results show that abatement over the next few years is economically valuable if there is a significant probability of having to stay below ceilings that would otherwise be reached within the characteristic timescales of the systems producing greenhouse gases. With continuing growth in CO₂ emissions akin to the IS92a scenario, CO₂ concentrations are likely to exceed 500 p.p.m., equivalent to at least a doubling of preindustrial CO₂ levels even if other greenhouse-gas emissions are strongly controlled¹² within about 50 years. If there is a significant risk that we need to stay below this level, or if the 'business as usual' scenario is significantly higher over coming decades than the IS92a baseline we have assumed, then the socioeconomic inertia in energy systems in itself suggests that delay in abatement efforts may prove costly. □

Received 9 January; accepted 24 October 1997.

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Acknowledgements. We are grateful to the CNRS, ADEME, the French Ministry of Environment, the Ministry of Education and Research for supporting the Oikia Programme, and to Shell, BP, Amerasia Hess and Asland Oil for supporting the Belgrave Fellowship of RIIA's Energy and Environmental Programme.

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The uniform and low ³He/⁴He ratios of HIMU basalts as evidence for their origin as recycled materials

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Several hypotheses have been proposed for the origin of the group of lavas having the isotopic signature known as 'high μ ' (HIMU, where $\mu = {}^{238}\text{U}/{}^{204}\text{Pb}$)^{1–4}; these explanations have invoked processes involving recycled oceanic crust and sediment, metasomatically enriched subcontinental lithosphere, or intra-mantle metasomatism^{1–12}. Here we present helium isotope analyses of HIMU basalts, with ages of 10–18 Myr, from three islands of the Cook–Austral Archipelago in the southern Pacific Ocean. We find that the HIMU samples have a relatively uniform and low ³He/⁴He

ratio of $6.8 \pm 0.9 R_A$ compared with mid-ocean-ridge basalt, whereas samples of other enriched-mantle lavas from this region have more variable and higher signatures. The consistency of our HIMU results with those obtained from previous analyses of HIMU lavas at St Helena¹³ in the Atlantic Ocean lead us to conclude that a relatively low and uniform $^3\text{He}/^4\text{He}$ ratio represents a general characteristic of the mantle source region for HIMU lavas. Also, the uniform $^3\text{He}/^4\text{He}$ ratio (in both space and time) suggests that recycled oceanic crust and/or sediments are present in the source region for HIMU lavas, as it seems less likely that the other candidate processes, invoking metasomatism, would produce such consistent values.

The low- $^3\text{He}/^4\text{He}$ signature observed in HIMU and EM-OIBs (enriched-mantle ocean island basalts)^{13–15} had previously been considered to document the residence of recycled materials in the mantle. However, a significant objection to this idea was raised by the example of Heard Island¹⁶ where reduction of $^3\text{He}/^4\text{He}$ with changing Nd isotope ratio has been observed by extensive sampling. Based on such observations, they claimed that possible contamination of crustal and/or lithospheric materials to OIBs with low $^3\text{He}/^4\text{He}$ might not be excluded, because of sampling problems such as the limited data set and the use of clinopyroxene (cpx) analyses alone. To solve this problem and obtain some additional constraints for HIMU, we have undertaken noble-gas analyses for HIMU samples from three islands in the Polynesian region.

Samples were selected from lavas from the Cook–Austral Archipelago and Society Archipelago in the French Polynesia. HIMU samples were chosen from Mangaia, Rurutu (ancient series) and Tubuai Islands in the Cook–Austral Archipelago^{2–4}, whose ages are around 18 Myr, 12 Myr and 10 Myr, respectively¹⁷. For comparison, EM1 samples from Rarotonga Island² (Cook–Austral Archipelago) and EM2 samples from Tahiti and Moorea Islands¹⁸ (Society Archipelago) were also analysed. Rurutu Island had binary activities at around 12 Myr (ancient series) and 1 Myr (recent series)¹⁷. Ancient series lavas are classified as HIMU, whereas recent series lavas show smaller Pb isotope ratios than ancient series lavas¹⁹.

Olivine and cpx phenocrysts were separated from ankaramites and olivine basalts for noble-gas analyses. The size of the phenocrysts used as samples was 1–3 mm. The samples were crushed for gas extraction in vacuum. Noble-gas analyses of He, Ne, Ar, Kr and Xe isotopes were made with a sector-type mass spectrometer (VG5400) at the Earthquake Research Institute, University of Tokyo. Helium was separated from neon by cryogenic pump before helium analysis. Helium-4 and helium-3 beams were measured by Faraday cup and by an electron multiplier ion counting system, respectively. The total blank for ^4He is less than $1 \times 10^{-11} \text{ cm}^3 \text{ STP}$. The helium isotope ratio was calibrated by

repeated analyses of standard gas from Kaminoyama Well, Yamagata, Japan ($6.04 \pm 0.29 R_A$ where R_A is the atmospheric helium ratio). The helium isotope results are shown in Table 1, and results for the other noble-gas isotopes will be reported elsewhere.

The result indicates a relatively uniform $^3\text{He}/^4\text{He}$ of $6.8 R_A$ ($\pm 0.9 R_A$; 1σ) for ten samples from three HIMU islands whose ages range from 10 Myr to 18 Myr (Fig. 1). The $^3\text{He}/^4\text{He}$ values of HIMU are slightly lower than those of Pacific mid-ocean-ridge basalt (MORB) ($8.7 R_A$)²⁰ and MORB worldwide ($8.3 R_A$)²¹, and they are independent of ^4He concentration. No systematic difference of $^3\text{He}/^4\text{He}$ is found between olivine and cpx samples. A result previously reported for Tubuai Island ($7.1 R_A$)¹³ is included within the range of present results.

EM samples from the Cook–Austral Archipelago (Rarotonga and Rurutu recent series) have similar $^3\text{He}/^4\text{He}$ to those of Pacific MORB, and samples from the Society Archipelago (EM2) show variable and higher $^3\text{He}/^4\text{He}$ than Pacific MORB. They show different characteristics from those of HIMU (Fig. 1). It is noteworthy that samples of the Rurutu ancient series and recent series show different $^3\text{He}/^4\text{He}$ as well as Pb isotope ratios¹⁹.

Figure 2 summarizes the $^3\text{He}/^4\text{He}$ data from HIMU and EM-OIBs worldwide, including the present result. Only data obtained by the crushing method are compiled. St Helena, another HIMU island in the Atlantic Ocean, has similar (but slightly lower) $^3\text{He}/^4\text{He}$ values to those of Polynesian HIMU samples. EM-OIBs, on the other hand, show rather variable $^3\text{He}/^4\text{He}$ values. EM-OIBs in the Atlantic and Indian Oceans, except for Heard Island, show lower $^3\text{He}/^4\text{He}$ than MORB, whereas $^3\text{He}/^4\text{He}$ values higher than those of MORB are generally found in Pacific EM-OIBs.

The following arguments indicate that slightly lower $^3\text{He}/^4\text{He}$ values than those of MORB can be assigned as a typical character for HIMU sources. (1) HIMU samples from both the Polynesian region and St Helena in the Atlantic Ocean show lower $^3\text{He}/^4\text{He}$ than MORB. (2) So far as the Southern Pacific region is concerned, only HIMU-OIBs have lower $^3\text{He}/^4\text{He}$ values, whereas EM-OIBs have rather higher $^3\text{He}/^4\text{He}$ than MORB. (3) The $^3\text{He}/^4\text{He}$ ratio of HIMU is uniform for samples from three islands of different ages (10–18 Myr). These findings exclude the possibility of crustal contamination raised by Hilton *et al.*¹⁶. Thus, helium isotopic evidence requires higher time-integrated $(U + \text{Th})/^3\text{He}$ for HIMU than for the MORB source. Further, only lavas from three islands with ages of 10–18 Myr in a concentrated area show the HIMU signature; these islands are surrounded by EM islands in the Southern Pacific. This indicates that the distribution of HIMU is quite limited both in time and space, implying that its source might be rather small compared with other sources such as EM components.

There are several candidates for the source material for HIMU

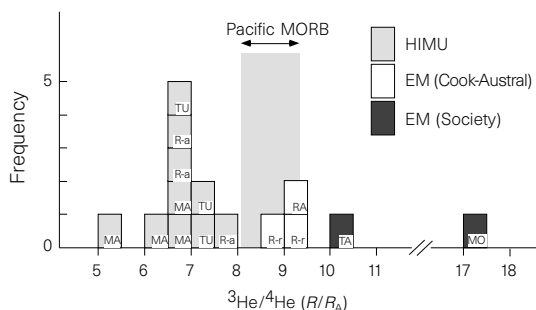


Figure 1 Frequency of helium isotope ratios obtained in this study for samples for different islands. The $^3\text{He}/^4\text{He}$ is normalized to the atmospheric $^3\text{He}/^4\text{He}$ ($1 R_A = 1.4 \times 10^{-6}$). The range of $^3\text{He}/^4\text{He}$ of Pacific MORBs ($8.68 \pm 0.63 R_A$)²⁰ is shown for comparison. Localities of samples are indicated as follows: MA, Mangaia; TU, Tubuai; R-a, Rurutu ancient series; R-r, Rurutu recent series; RA, Rarotonga; MO, Moorea; TA, Tahiti.

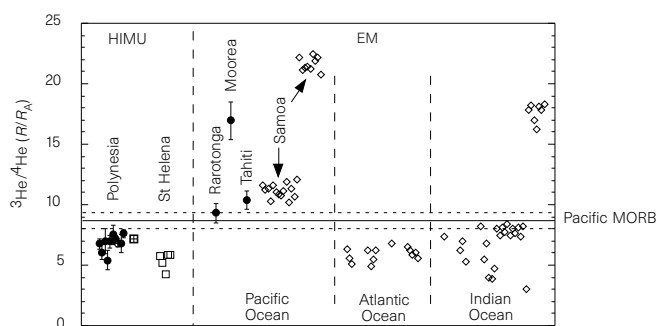


Figure 2 The $^3\text{He}/^4\text{He}$ ratios for HIMU and EM samples from the Pacific, Atlantic and Indian Oceans. Only data obtained by the crushing method are compiled. Solid symbols are data from this study. Open symbols represent data from Tubuai, St. Helena, Samoa, Jan Mayen, Tristan da Cunha, Gough, Prince Edward, Canary, Kerguelen and Heard Island^{13–16,30}. Error bars (1σ) are shown only for our data.

with elevated $(U + Th)/^3He$, such as (1) metasomatically enriched subcontinental lithosphere^{6,8}, (2) ancient subducted oceanic crust^{3-5,10,12}, and (3) intra-mantle metasomatism^{2,7,9,11}. The first model has been evaluated using helium isotopes in the case of the subcontinental lithosphere of the southwestern United States²¹. It was suggested that metasomatic processes, which occurred in the Proterozoic era, slightly increase the U/He of metasomatized subcontinental lithosphere. Accordingly, variation of $^3He/^4He$ between a MORB-like value ($\sim 8R_A$) and a slightly radiogenic value ($\sim 5R_A$) is observed in magmas derived from the subcontinental lithosphere. On the other hand, Dunai and Baur²² proposed a model of enriching process in which addition of dehydrated oceanic crust would increase the $(U + Th)/^3He$ of the subcontinental lithosphere because helium would be completely removed from the subducting slab by dehydration^{23,24}. Following similar logic, the second possibility of oceanic crust being directly recycled into the mantle would be also a candidate for producing elevated $(U + Th)/^3He$ through dehydration. In both cases considered above, the processes that elevate $(U + Th)/^3He$ would have taken place near to the Earth's surface and the material with $(U + Th)/^3He$ would have been recycled back into the source region of HIMU-type mantle plume.

The third model, that of intra-mantle metasomatism, requires the generation of metasomatic melts derived from some portions of the mantle that increase $(U + Th)/^3He$ to produce the HIMU source. The metasomatic models (1) and (3) might be further tested by looking at some constraints such as the uniformity of $^3He/^4He$ or the Sr and Nd isotopic ratios of HIMU^{2,3,19}. Models (1) and (3) require a uniformly metasomatized region whose volume is large enough to be the source of OIBs¹². Although subcontinental lithosphere might be uniformly metasomatized and enriched by subduction-related processes^{21,22}, the intra-mantle metasomatic model further requires the special conditions of a relatively limited range of elevated $(U + Th)/^3He$ and radiogenic ingrowth time of $^3He/^4He$. However, we have no information about these conditions at present. More detailed knowledge of the character of $^3He/^4He$ and $(U + Th)/^3He$ for metasomatic fluids and melts derived from

various regions of the mantle and the subducted material is needed to evaluate these models.

Concerning the model of oceanic crust recycling, the problem is that characteristic $^3He/^4He$ values of $\sim 6.8R_A$ for HIMU seem to be too high to be explained by the addition of *in situ* radiogenic 4He in recycled materials. The $^3He/^4He$ of recycled oceanic crust would be reduced from $8R_A$ to $5R_A$ in only 100 Myr, owing to 4He production from U and Th (ref. 13). As the HIMU source is estimated to have formed at about 2 Gyr from Pb isotope ratios^{2,3,12}, recycled materials should have a much lower $^3He/^4He$ ($< 1R_A$).

However, the diffusive loss of radiogenic 4He from recycled materials can be raised as a possible explanation of the $^3He/^4He$ values of HIMU, because diffusion of helium would be much faster than that of the other elements. Provided that the recycled materials resided at the boundary between the upper and lower mantle, where the temperature is around 1,600 °C, volume diffusion of helium in olivine is estimated to be around $2 \times 10^{-10} \text{ m}^2 \text{ s}^{-1}$ (from extrapolation of diffusion data for olivine to 1,600 °C; ref. 25). However, grain boundary diffusion might be the controlling process, rather than volume diffusion. Although the grain boundary diffusion coefficient is not well known, Trull and Kurz²⁶ have suggested that grain boundary diffusion of helium would not be faster than the diffusion in melts and fluids. In this case, $1 \times 10^{-8} \text{ m}^2 \text{ s}^{-1}$ for the helium diffusion coefficient (from extrapolation of diffusion data for a basaltic melt to 1,600 °C; ref. 27) is the maximum estimate if grain boundary diffusion controls helium mobility. Then, the diffusion length of helium in 1 Gyr is about 5 km and 35 km for volume diffusion and grain boundary diffusion, respectively. Consequently, radiogenic 4He produced from U and Th would not be trapped in ancient recycled materials.

Hence, an open-system evolution model of $^3He/^4He$ in recycled materials can be envisaged as follows. Assuming that recycled material subducted as part of a slab would be surrounded by the upper mantle material and that helium would diffuse between them, $^3He/^4He$ in the recycled material can be simulated as a function of storage time, taking into account the effects of helium diffusion and

Table 1 Helium isotope ratios of HIMU and EM samples

Sample	Rock type	Mass (mg)	Phase*	$^{[He]}$ $10^{-9} \text{ cm}^3 (\text{STP}) \text{ g}^{-1}$	$R/R_A^\dagger$
Cook-Austral Archipelago					
<i>Rarotonga</i>					
RTG-C4	ankaramite	1,303	cpx	3.6	9.3 ± 0.8
RTG-C5	ankaramite	1,248	ol	$< 0.3^\ddagger$	-
		797	cpx	$< 0.3^\ddagger$	-
RTG-C8	olivine basalt	1,395	cpx	$< 0.3^\ddagger$	-
RTG-C10	ankaramite	1,383	cpx	$< 0.3^\ddagger$	-
<i>Mangaia</i>					
MGA-C8	ankaramite	1,067	cpx	5.6	6.8 ± 0.4
MGA-C12	ankaramite	564	ol	8.0	6.1 ± 0.6
MGA-C23	ankaramite	1,328	cpx	3.6	7.0 ± 1.0
MGA-C29	ankaramite	515	ol	12.1	5.4 ± 0.8
<i>Tubuai</i>					
TU01-01	ankaramite	1,490	cpx	13.9	6.9 ± 0.5
TU09-01	ankaramite	1,303	cpx	2.7	7.5 ± 0.8
TU12-01	ankaramite	1,300	cpx	189.2	7.2 ± 0.3
<i>Rurutu (ancient series)</i>					
RU01-01	olivine basalt	728	ol	16.2	6.8 ± 0.3
RU02-01	ankaramite	1,091	cpx	8.6	6.8 ± 0.8
RU09-01	ankaramite	1,542	ol	37.8	7.6 ± 0.4
<i>Rurutu (recent series)</i>					
RRT-C31	olivine basalt	767	ol	4.3	9.5 ± 1.2
RRT-C33	olivine basalt	1,517	ol	2.2	8.9 ± 1.1
Society Archipelago					
<i>Moorea</i>					
MO01-01	ankaramite	852	ol	1.3	17.0 ± 1.6
<i>Tahiti</i>					
TA01-02	ankaramite	1,490	cpx	1.6	10.4 ± 0.8

* Olivine (ol) and clinopyroxene (cpx) were crushed in vacuum to release noble gases.

† $^3He/^4He(R)$ is normalized by the atmospheric $^3He/^4He$ ($R_A = 1.4 \times 10^{-6}$). All errors are 1σ .

‡ $^3He/^4He$ was not determined owing to scarcity of helium extracted from samples.

radiogenic ^4He production from U and Th. The initial concentrations of ^3He and ^4He in the recycled material and surrounding mantle are assumed to be uniform for simplicity.

The preliminary results of such a simulation indicate that, under the constraint that the $^3\text{He}/^4\text{He}$ of recycled material is $6.8R_A$, its thickness should be about 2 km, given a diffusion coefficient of $1 \times 10^{-8} \text{ m}^2 \text{ s}^{-1}$, a storage time of 1 Gyr and U concentration of 70 p.p.b. which is equivalent to U in fresh MORB²⁸. Changing the model parameters of U concentration from 70 to 320 p.p.b. (altered MORB)²⁹ and the storage time from 1 to 3 Gyr leads to estimates for the thickness of recycled material ranging from 0.2 km to 3 km. Accordingly, the open system model for helium indicates that the thickness of recycled material as a portion of a slab would be in the order of 1 km, which is consistent with the thickness of recycled material for a HIMU source. □

Received 5 March; accepted 15 September 1997.

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Acknowledgements. We thank T. Kogiso for samples from Mangaia and Rarotonga Islands, R. Maury for his help during our sampling in Rurutu and Tubuai Islands, and K. Nagao for guidance on technical aspects of the mass spectrometry. Comments from D. Graham were helpful in improving the manuscript. This study was supported in part by the Grant-in-Aid for Scientific Research from the Ministry of Education, Science and Culture, Japan to I.K.

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The seed-storing corvid Clark’s nutcracker learns geometric relationships among landmarks

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Many animals regularly return to particular locations such as hives, nests, wintering grounds or cache sites. This ability clearly implies that animals possess information that allows them to find a route from their current location to their goal. However, the nature of this information is, in many cases, unknown. One particularly important issue is whether this information encodes at least some of the geometric relationships among real-world objects, which would meet a strict definition of a cognitive map^{1,2}. Are animals sensitive to such geometric relationships? Although there is clear evidence that animals can learn vectors that represent a goal location in terms of absolute distance and direction to a landmark, there is little evidence of any ability to extract abstract geometric rules^{3–8}. Here we report data demonstrating that the corvid Clark’s nutcracker (*Nucifraga columbiana*) can learn to find the point halfway between two landmarks that vary in the distance that separates them. This learning is based on a general principle, as the birds correctly find the halfway point when the landmarks are presented with new distances between them. This demonstrates the ability to find a point defined not by the relationship between a goal and a landmark but by the relationship between landmarks. Further experiments demonstrate that there were two distinct processes involved in locating the halfway point, the use of directional bearings to find the (hypothetical) line connecting the landmarks and finding the correct place along that line.

Five wild-caught Clark’s nutcrackers, maintained at 90% of their free-feeding weights, were tested in a 4.4×2.7 m observation room

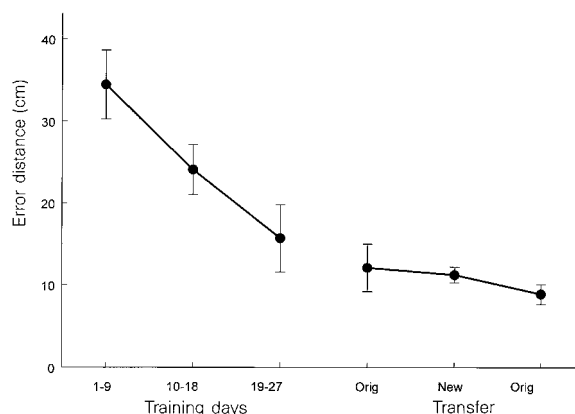


Figure 1 Mean distance ($\pm$ s.e.) between each of the first 5 digs and the goal location on test trials throughout training and the transfer test. During training and the first and last blocks of transfer inter-landmark distances were 20–120 cm in 20-cm increments. During the second block of transfer, inter-landmark distances of 30–110 cm in 20-cm increments were tested. The overall decline in error scores was significant ($F_{2,8} = 6.27$, $P < 0.05$) and there were no significant differences among the three blocks of trials of the transfer test ($F_{2,8} = 1.08$, $P < 0.25$).

with various posters on the walls, and a door, porthole and smoked-glass window on the east wall. During the first 27 days of the experiment, each bird entered the room through the porthole four times. There were two 40-cm-high landmarks (green and yellow PVC pipes) that were always placed north and south of each other, with the yellow landmark to the north. On the first three trials each day, a seed was partially buried (but still visible) in the cellulose substrate on the floor halfway between the landmarks. On the fourth trial, the test trial, the seed was fully buried 1 cm below the surface halfway between the landmarks. The distance between the landmarks varied from 20 to 120 cm in 20-cm increments in random order across trials. In addition, both the east–west and north–south locations varied so that there were 135 different locations in the room that served as the correct location across trials. Testing continued until either the bird found the seed, made 50 unsuccessful digs or did not dig for 10 min, and the location of each of the first 5 digs was determined from videotape.

During the next 45 trials (transfer test) and all subsequent testing, the landmarks were presented in the same north–south orientation, all trials were conducted with the seed completely buried, and 2–4 test trials were conducted per day. For the first and last blocks of 10 trials during these 45 trials, the landmarks were presented with the same inter-landmark distances as during training. During the middle block of 25 trials, the birds were tested with 5 new distances (from 30–110 cm, in 20-cm increments, in random order) to test the ability of the birds to generalize to new inter-landmark distances.

The birds readily learned to bisect the inter-landmark distance and continued to do so when presented with the new transfer distances (Fig. 1). We also analysed the position of digs on the north–south axis during the transfer test. If the birds learned a general bisection principle, the distribution of digs around the point of bisection should be the same for the original training distances and the new transfer distances on the first day of testing at each new distance. These distributions were virtually identical (Fig. 2), with peak locations of digging just to the south of the point of bisection during both conditions (probably due to the position of the porthole at the southern end of the wall).

These results demonstrate that the nutcrackers learned to use a general principle based on the geometric relationship between the landmarks. In an attempt to understand the decision processes involved, we broke down the data from the transfer test into two components: error along the north–south line connecting the

landmarks and error perpendicular to that line. Results indicated that two processes were involved. Error along the line was greater than error perpendicular to the line and the precision of placement perpendicular to the line appeared to be more independent of inter-landmark distance than precision along the line (Fig. 3).

These differences as a function of axis of analysis suggest that the process of finding the line connecting the landmarks was independent of finding the correct location along the line. We used probe trials with manipulation of the landmarks to test possible mechanisms for these two processes. Throughout this probe testing, birds received four buried seed trials per day, with the landmarks located 50, 70, 90 or 110 cm apart (in random order). For half the days, chosen at random, the 90-cm trial was a control trial with the landmarks in the position used throughout training; on the other half, it was a probe trial (described below).

The two most obvious ways to position oneself on the line (as opposed to off the line) are (1) by taking bearings (direction) to both landmarks and comparing them or (2) because the landmarks were always located north and south of each other, by taking a bearing from one of the landmarks. We tested these possibilities by using probe tests in which the landmarks were rotated from the north–south orientation. The amount of rotation was approximately 15°; each landmark was displaced 10 cm off the north–south line. Testing was carried out over 8 days (4 control, 2 clockwise rotation, 2 anticlockwise in random order). If a comparison of bearings to the landmarks was used, the birds should continue to dig halfway between the two landmarks (on the new rotated line between the landmarks); if a single bearing was used, digging should be displaced off the line. The birds tended to dig south of the green landmark in both experimental conditions (Fig. 4a), but the effect was small, certainly less than the 10-cm displacement predicted by the use of a single bearing. It appears that the birds were using both a comparison of bearings and a single bearing from the green landmark, compromising between the two when they were in conflict. To test this idea further, we calculated the distance between the average position of the first five probes of each bird during each trial and both the line connecting the rotated landmarks and the north–south lines from the individual landmarks. We then compared the distance to the rotated line with the distance to whichever north–south line came closest to the dig position.

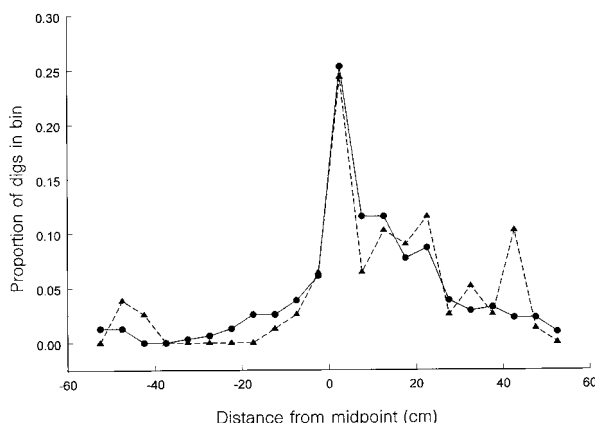


Figure 2 Frequency distribution of the location of digs in the north–south axis during transfer testing. Circles, Distribution for original training distances, taken from first and last blocks of transfer. Triangles, Distribution for new distances, taken from the first test at each new distance during the second block of transfer. These distributions do not differ (χ^2 test, $P > 0.25$).

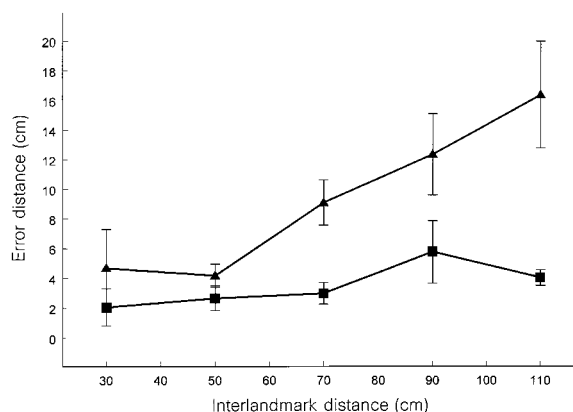


Figure 3 Mean distance ($\pm$ s.e.) between each of the first five digs and the goal location as a function of inter-landmark distance and axis of analysis. Triangles, Distance along the north–south line connecting the two landmarks; Squares, distance in the east–west axis. The magnitude of error in the north–south axis was greater than that in the east–west axis ($F_{1,4} = 28.86$, $P < 0.01$) and increased with greater inter-landmark distances ($F_{4,16} = 4.34$, $P < 0.02$) whereas there was a tendency for the precision of placement perpendicular to the line to be relatively independent of inter-landmark distance but precision along the line became less accurate as inter-landmark distance increased ($F_{4,16} = 2.52$, $P = 0.08$).

These distances did not differ significantly, averaging 4.72 and 5.45 cm, respectively.

The most obvious way to position oneself at the correct distance along the line is by matching some aspect of the projected retinal image of the landmarks. The most obvious aspect to match would be the apparent height of the landmarks. We tested this possibility by manipulating the height of the landmarks. Testing was carried out over 16 days (8 control, 4 in which one landmark was 56.5 cm high, 4 in which one landmark was 23.5 cm high, with each landmark being manipulated equally often, and in random order). If matching visual input from the landmarks was being used, then making a landmark smaller should cause the bird to move closer to that landmark and *vice versa*. However, there were no effects of changing the size of the landmarks ($F_{4,16} = 2.20$, $P > 0.10$). These results are in marked contrast to those obtained with bees⁸, but leave

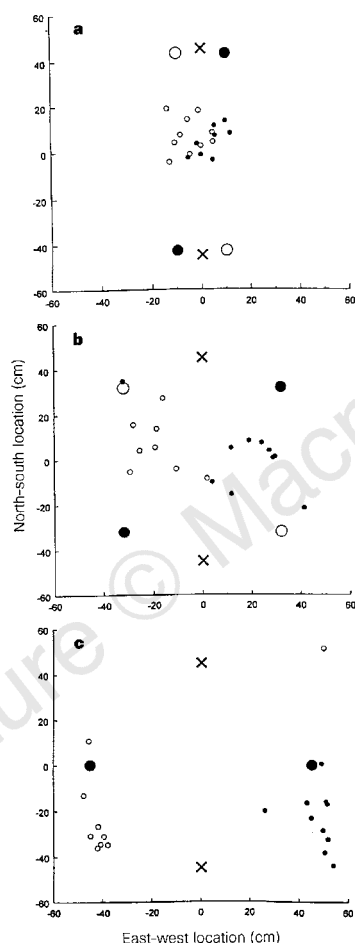


Figure 4 **a**, Map showing the location of probing during the 15° rotation testing. Black circle, Positions of landmarks during the clockwise rotation; empty circle, positions of landmarks during the anticlockwise rotation; cross, positions of landmarks during control trials. Each small filled circle represents the mean position of the first five probes of a single clockwise test session; each small empty circle represents the mean position of the first five probes for a single anticlockwise test session. The halfway point was always at 0,0. One bird (603) is omitted because it searched next to the east wall (outside the range of the figure). **b**, Identical to **a**, except showing the results of the 45° rotation of the landmarks. Two sessions are not shown, one because the bird dug directly under the green landmark during anticlockwise testing, the other because the bird (601) dug next to the east wall. **c**, Identical to **a**, except showing the results of the 90° rotation of the landmarks (note that clockwise and anticlockwise rotations led to the landmarks being in the same location, green on the right for clockwise and on the left for anticlockwise). One session is omitted because the bird (601) searched next to the east wall.

open the possibility of retinal matching based on some other aspect of the landmarks (such as width or base).

Although a number of previous studies^{9–11} have shown that nutcrackers do not use smell or other cues emanating directly from buried pine seeds, we conducted an explicit test of this possibility. Testing was conducted over 8 days; 4 control days with seeds present and 4 test days during which, on the 90-cm test trial, there were no seeds present (in random order). In analysing these data, we only used the positions of the first two digs per trial because the birds often found the buried seed within 3 digs on control trials. There were no significant effects of seed removal on the accuracy of digging: the average error distance was 7.1 cm (± 0.9 s.e.) on seed trials and 10.9 (± 1.6 s.e.) cm on no-seed trials ($F_{1,4} = 3.18$, $P > 0.15$).

We next tested the effects of large rotations of the landmark array by giving probe tests during which the landmarks were rotated either 45° or 90°, in both clockwise and anticlockwise directions. Testing was conducted over 16 days (8 control tests, 4 tests each at 45° and at 90°, in random order). The effects of the 45° rotation were similar to the effects of the 15° rotation (Fig. 4b). The birds appeared to compromise between using a single bearing from one of the landmarks and using a comparison of bearings from both landmarks. An analysis of distances between digging locations and bearing lines again showed that there were no significant differences in the distance from point of digging to the rotated line (11.68 cm) and to the closest absolute bearing line (15.00 cm). However, when the landmarks were rotated 90°, the birds depended on single bearings (Fig. 4c). In most cases, the birds dug directly south of the green landmark; in the remainder, they dug directly north of the yellow landmark. The average distance to the rotated line connecting the landmarks was 26.57 cm, whereas the average distance to the closest north–south line was 5.08 cm ($F_{1,4} = 65.19$, $P < 0.01$). Thus, as the degree of rotation of the landmarks increased from 45° to 90°, the birds appeared to shift to using absolute bearings from a single landmark. It should be noted that throughout all rotational probe tests, especially the 45° and 90° tests, all birds tended to dig closer to one landmark (usually the green landmark) than the other. This indicates that the birds could not have been using a judgement of equal distance to the landmarks (including matching of any aspect of the retinal images). However, it is possible that the rotation of the landmarks led to a breakdown in the process normally used to determine the correct location along the line connecting the landmarks.

Many experiments have demonstrated that animals can learn to find a goal that bears a fixed relationship to moveable landmarks (refs 3, 4, 11–13 and see also refs 5, 6 for evidence that landmark learning in rats is best when landmarks are stable in space). At the neural level, many place cells in rat hippocampus have been found to be responsive to absolute distance from an environmental feature, although some cells responded instead to the proportion of the distance between two walls¹⁴. In the present experiment, the distance between the goal and the landmarks varied from trial to trial according to the distance between the landmarks. The results clearly demonstrate that nutcrackers can learn to find a spatial position defined by an abstract geometric relationship. They are consistent with the hypothesis that animals are capable of representing the geometric relationships among landmarks as required by a strict definition of the cognitive map¹. □

Received 31 March; accepted 25 August 1997.

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Acknowledgements. We thank K. Cheng, S. Shettleworth, S. Yoerg, J. Templeton, A. Bond, K. Gould-Beierle, B. Gibson and C. Cink for comments on previous drafts of this paper, and D. W. Stephens for assistance with data analysis. The research was supported by The National Science Foundation and the Howard Hughes Medical Institute.

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The capacity of visual working memory for features and conjunctions

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Short-term memory storage can be divided into separate sub-systems for verbal information and visual information¹, and recent studies have begun to delineate the neural substrates of these working-memory systems^{2–6}. Although the verbal storage system has been well characterized, the storage capacity of visual working memory has not yet been established for simple, supra-threshold features or for conjunctions of features. Here we demonstrate that it is possible to retain information about only four colours or orientations in visual working memory at one time. However, it is also possible to retain both the colour and the orientation of four objects, indicating that visual working memory stores integrated objects rather than individual features. Indeed, objects defined by a conjunction of four features can be retained in working memory just as well as single-feature objects, allowing sixteen individual features to be retained when distributed across four objects. Thus, the capacity of visual working memory must be understood in terms of integrated objects rather than individual features, which places significant constraints on cognitive and neurobiological models of the temporary storage of visual information⁷.

To measure the capacity of working memory for simple features, we used a variant of the sequential comparison procedure developed by Phillips⁸. Subjects viewed a sample array and a test array on each trial, separated by a brief delay, and then indicated whether the two arrays were identical or differed in terms of a single feature. The accuracy of this discrimination was assessed as a function of the number of items in the stimulus array (the set size) to determine how many items could be accurately retained in working memory. In addition, control experiments were conducted to ensure that performance truly reflected the capacity of visual working memory and was not influenced by verbal working memory or by limitations in perception, memory encoding, or decision processes.

The first set of experiments examined working memory capacity for simple colours (Fig. 1a). The sample array consisted of 1–12 coloured squares and was presented for 100 ms. This was followed by a 900-ms blank delay interval and then a 2,000-ms presentation of the test array, which was either identical to the sample array or differed in the colour of one of the squares. Performance was nearly

perfect for arrays of 1–3 items and then declined systematically as the set size increased from 4 to 12 items. According to the method for estimating memory capacity described by Pashler⁹, these data indicate that the observers were able to retain the colours of roughly four items in working memory, which is similar to previous estimates for alphanumeric characters²¹.

To demonstrate that this estimate of capacity accurately reflects limitations in visual working memory with no significant contribution from verbal working memory, we tested the effects of adding a verbal memory load. In half of the trial blocks, the observers were presented with two digits before each sample array and were required to hold these digits in memory and then say them aloud at the end of the trial. Adding a verbal load did not significantly alter performance on the colour task (Fig. 1a), indicating that our capacity estimate was not influenced by verbal working memory.

It was also necessary to demonstrate that the relatively small memory capacity observed in this experiment was not a result of limitations in processes other than working-memory storage. To rule out limitations in perceiving the stimuli and encoding them in working memory, we varied the duration of the sample stimulus, comparing the original 100-ms duration with a 500-ms duration. This allowed substantially more time for perceiving the stimuli and encoding them in memory, which should have led to improved performance if these were limiting factors. However, performance was not significantly influenced by variations in sample duration (Fig. 1b), indicating that the errors at set sizes of 4–12 reflected limitations in storage capacity rather than limitations in perceiving or encoding the stimuli.

We next examined the possibility that performance was limited by decision factors. At larger set sizes, more decisions must be made, and this can lead to an increase in errors even in the absence of any capacity limitations^{10,11}. To rule out this explanation, we conducted an experiment in which the memory requirements were the same as in the original experiment but only a single decision was necessary, regardless of the set size. Specifically, we used a partial report procedure in which we cued the observers to make a decision about only one of the items in the test array by presenting an outline box around the one item that might have been different from the sample array. This required them to retain information from all of the items in the sample array, but allowed them to restrict decision processes to a single item in the test array. As shown in Fig. 1b, this manipulation did not significantly alter performance, indicating that accuracy was not limited by decision factors (or, alternatively, that the subjects were unable to use the cue box effectively, which seems unlikely given that previous studies have found similar cues to be very effective in improving performance in decision-limited tasks^{12,13}).

To determine whether capacity is different for different feature dimensions, memory for orientation was compared with memory for colour using 4, 8 or 12 bars that varied both in colour and in orientation. The observers were instructed to detect either colour changes or orientation changes (in different trial blocks), and a verbal load was used in both cases. The effects of set size on accuracy were nearly identical for colour and orientation, with a capacity of about four items for both feature types.

We then assessed whether visual information is stored in working memory as individual features or as integrated objects. This was tested by comparing memory for simple features with memory for objects defined by a conjunction of features. Observers performed the same sequential comparison task used above (while performing a concurrent verbal load task) with arrays of 2, 4 or 6 coloured bars of varying orientations. Relatively small set sizes were used so that the objects could be widely spaced, which was necessary to avoid 'illusory conjunctions' in the perception of the bars¹⁴. In one condition, only colour could vary between the sample array and the test array, and the observers were instructed to look for a colour change. In a second condition, only orientation could vary, and the

observers were instructed to look for an orientation change. In the third and critical condition, either colour or orientation could vary, and the observers were required to remember both features of each object. In this last condition, accurate performance with a set size of four objects would require the observer to retain eight features (four colours and four orientations), whereas only four features would be required for accurate performance in the simple feature conditions. Performance was essentially identical for the feature and conjunction conditions despite the greater total number of features that had to be retained in the conjunction condition (Fig. 1c). This indicates that visual working memory stores integrated object percepts rather than individual features, just as verbal working memory can store higher-order 'chunks'¹⁵. This is also analogous to findings from visual attention experiments, which have shown that attention is directed to entire objects rather than to individual features and that, consequently, two features of a given object can be reported as accurately as a single feature¹⁶.

Because the stimulus arrays shown in Fig. 1c always varied in both colour and orientation, it is possible that the subjects were unable to avoid encoding both features even when only one feature was relevant. To rule out this potential explanation of the similar results obtained for the feature and conjunction conditions, a second version of this experiment was conducted in which the irrelevant feature dimension was held constant in the single-feature conditions (all of the rectangles were black when the subjects were required to remember orientation and all were vertical when the subjects were required to remember colour). The results were virtually identical to those shown in Fig. 1c, with statistically indistinguishable performance in the feature and conjunction conditions.

To extend these findings, we conducted an experiment in which

the objects were defined by a conjunction of four features: colour, orientation, size and the presence or absence of a gap. Performance was just as good in this quadruple conjunction condition as it was in the individual feature conditions (Fig. 1d), indicating that 16 features distributed across 4 objects can be retained as accurately as 4 features distributed across 4 objects.

The surprisingly good performance for conjunctions could be explained by the use of separate, independent memory systems for each feature type rather than the storage of integrated object representations. To rule out this possibility, we examined colour-colour conjunctions in which each object consisted of a large square of one colour and a small inner square of a different colour. Observers were just as accurate with these colour-colour conjunctions as they were with either the large outer squares or the small inner squares presented alone (Fig. 1e). Thus, eight colours distributed across four objects can be retained as accurately as four colours distributed across four objects. Because both features of each object consisted of colours, the high accuracy observed in the conjunction condition cannot be explained by the existence of independent memory systems for different features.

These results indicate that integrated object percepts are stored in visual working memory, leading to a large capacity for retaining individual features as long as the features are confined to a small number of objects. Although there may be limits on the number of features that can be linked together in a single object representation, our results indicate that at least four features can be joined in this manner with no cost in terms of storage capacity.

The present findings have important implications for both the nature of the input to, as well as the contents of, visual working memory. Specifically, studies of selective attention indicate that attentional processes are used to combine the features of an object

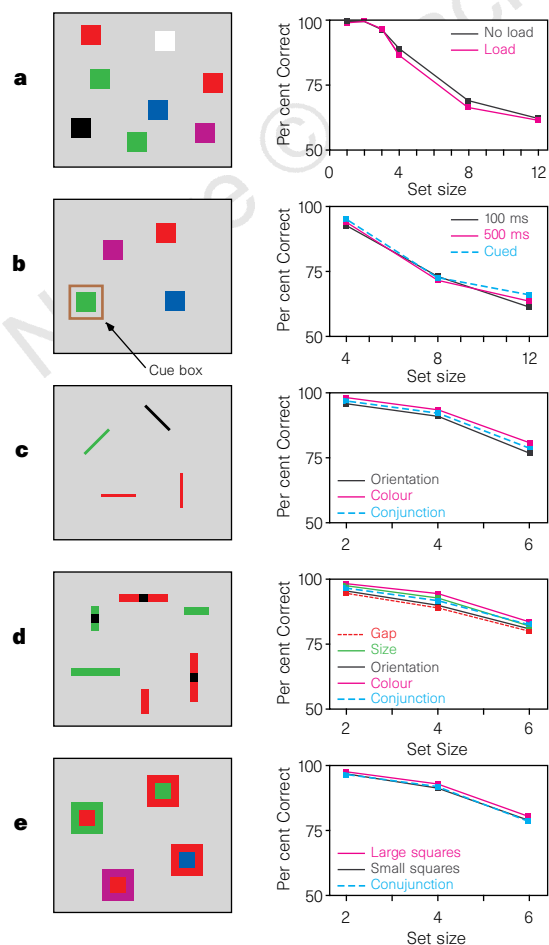


Figure 1 Example stimulus arrays (not drawn to scale) and performance on the sequential comparison task. All set size effects shown here were statistically significant at the $P < 0.001$ level (ANOVA). No other effects approached the $P < 0.05$ level of significance. **a**, Performance with and without a verbal load for simple colour stimuli. **b**, Comparison of 100-ms and 500-ms sample durations for simple colour stimuli (with a verbal load and no cue box). Also shown is the performance in a similar experiment with a cue box that indicated the one item that might have changed colour (100-ms sample duration and no verbal load). **c**, Comparison of performance when the observers were instructed to detect a colour change, an orientation change or a change in either feature (conjunction task). **d**, Comparison of performance for each of four simple features and the conjunction of all four features. **e**, Comparison of performance for colour-colour conjunctions versus the individual large and small squares.

into an integrated percept¹⁷, and it is these integrated object percepts that appear to be stored in visual working memory. Neurobiological accounts of working memory must therefore include a mechanism for keeping the features of an object bound together during the retention interval. A leading candidate mechanism is the use of oscillatory or temporally correlated firing patterns among the neurons that code the features of an object^{18–20}. Such a mechanism can also readily explain the relatively small number of objects that can be held in working memory concurrently: as the number of concurrent objects increases, the possibility of accidental correlations between neurons that code different objects also increases⁷. However, this would not necessarily place any limits on the number of features that can be bound together into a single object representation, which is consistent with our findings. □

Methods

Ten neurologically normal college students participated in each experiment. Each of these observers received 32–40 trials in each condition, where a condition consisted of a combination of set size and some other variable, such as the presence or absence of a verbal load.

All stimulus arrays were presented within a $9.8^\circ \times 7.3^\circ$ region on a video monitor with a grey background (8.2 cd m^{-2}), and the items in a given array were separated by at least 2.0° (centre to centre). One feature of one item in the test array was different from the corresponding item in the sample array on 50% of trials; the sample and test arrays were otherwise identical.

The experiments shown in Fig. 1a used sample arrays consisting of 1, 2, 3, 4, 8 or 12 coloured squares ($0.65^\circ \times 0.65^\circ$), each of which was selected at random from a set of 7 highly discriminable colours (red, blue, violet, green, yellow, black and white). The experiments shown in Fig. 1b used the same stimuli, but set size was limited to 4, 8 or 12 items.

The experiments testing combinations of colour and orientation (Fig. 1c) used arrays of $0.03^\circ \times 1.15^\circ$ rectangles, each of which was constructed by combining one of four orientations (vertical, horizontal, -45° and $+45^\circ$) with one of four colours (red, green, blue and black). The stimuli used in the experiment shown in Fig. 1d were combinations of horizontal or vertical, red or green, small or large ($0.13^\circ \times 1.0^\circ$ or $0.13^\circ \times 2.0^\circ$) and continuous or broken (broken by a 0.26° black gap).

The colour–colour conjunction stimuli shown in Fig. 1e consisted of a small square ($0.65^\circ \times 0.65^\circ$) embedded in a large square ($1.3^\circ \times 1.3^\circ$). The inner and outer colours for a given object were selected from the set of red, green, violet and blue with the constraint that the inner and outer colours were always different from each other. The simple feature conditions of this experiment used either the large squares presented alone or the small squares presented alone.

Received 16 June; accepted 20 August 1997.

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Acknowledgements. This research was supported by grants from the McDonnell-Pew Program in Cognitive Neuroscience and the National Institute of Mental Health.

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A role for the Ras signalling pathway in synaptic transmission and long-term memory

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Members of the Ras subfamily of small guanine-nucleotide-binding proteins are essential for controlling normal and malignant cell proliferation as well as cell differentiation¹. The neuronal-specific guanine-nucleotide-exchange factor, Ras-GRF/CDC25Mm (refs 2–4), induces Ras signalling in response to Ca^{2+} influx⁵ and activation of G-protein-coupled receptors *in vitro*⁶, suggesting that it plays a role in neurotransmission and plasticity *in vivo*⁷. Here we report that mice lacking Ras-GRF are impaired in the process of memory consolidation, as revealed by emotional conditioning tasks that require the function of the amygdala; learning and short-term memory are intact. Electrophysiological measurements in the basolateral amygdala reveal that long-term plasticity is abnormal in mutant mice. In contrast, Ras-GRF mutants do not reveal major deficits in spatial learning tasks such as the Morris water maze, a test that requires hippocampal function. Consistent with apparently normal hippocampal functions, Ras-GRF mutants show normal NMDA (N-methyl-D-aspartate) receptor-dependent long-term potentiation in this structure. These results implicate Ras-GRF signalling via the Ras/MAP kinase pathway in synaptic events leading to formation of long-term memories.

Several distinct mechanisms leading to Ras activation and initiation of the MAP kinase (MAPK) cascade have been elucidated⁸. Growth-factor receptors of the tyrosine kinase family activate Ras proteins by recruiting the ubiquitously expressed Sos exchange

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factors to the cell membrane through the adapter protein Grb2 (ref. 9). In neurons, an increase in intracellular calcium levels activates the Ras/MAPK pathway¹⁰.

The exchange factor Ras-GRF (also called CDC25Mm) is exclusively expressed in neurons of the postnatal and adult central nervous system (CNS)¹¹ and is mainly localized in the synaptosomal fraction¹². Following activation of muscarinic M1 and M2 receptors, Ras-GRF becomes phosphorylated and this increases its exchange activity⁶. Instead of presenting a Grb2-binding domain, Ras-GRF contains an ilimaquinone domain. When intracellular calcium is increased, this domain is necessary for binding to Ca^{2+} -calmodulin and for Ras-GRF-dependent activation of the Ras/MAPK pathway⁵.

To examine the role of Ras-GRF in the activity of the adult brain, we inactivated the mouse gene using homologous recombination in embryonic stem (ES) cells, by replacing the most 5' region encoding the exchange-factor catalytic domain with the phosphoglycerate kinase (PGK) promoter-driven neomycin cassette (Fig. 1a). Ras-GRF^{-/-} mice are viable and fertile. To characterize the mutant mice at the molecular level, we generated amino-terminal-specific antibodies against Ras-GRF. Using NIH3T3 cells expressing full-length Ras-GRF in immunoprecipitation assays, we showed that commercially available anti-C-terminal and our anti-N-terminal antiserum specifically recognized a single band of relative molecular mass (M_r) 140K, corresponding to the Ras-GRF gene product (Fig. 1b). Detection of p140 was competent with the corresponding antigen, the PHP fragment of Ras-GRF, further demonstrating the specificity of the N-terminal antibodies. We performed western blot analysis on brain extracts using the two different antisera. The N-terminal antibodies specifically and exclusively recognized p140^{Ras-GRF} only in mice carrying the wild-type allele, demonstrating that the introduced mutation resulted in the complete loss of the p140^{Ras-GRF} gene product (Fig. 1c). The same loss of signal was also demonstrated using the C-terminal-specific antibodies.

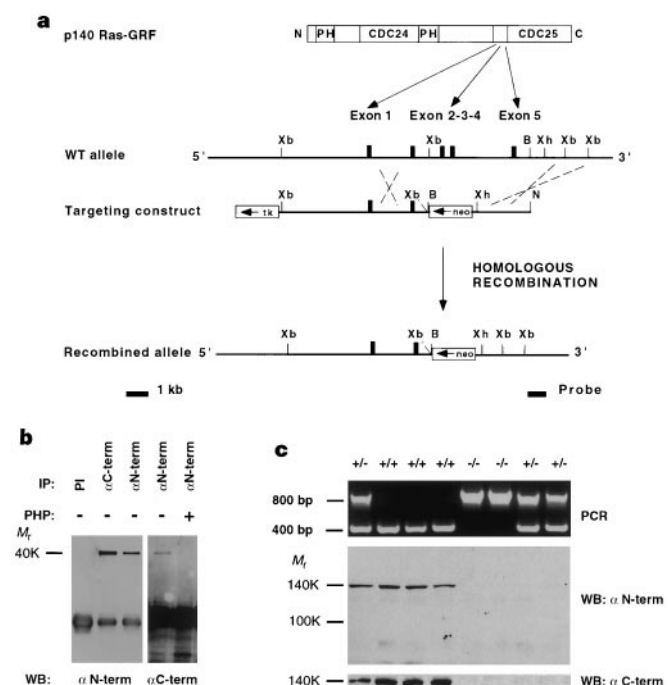
Interestingly, some of the Ras-GRF^{+/-} mice also lacked p140^{Ras-GRF} and by western blot analysis were indistinguishable from Ras-GRF^{-/-} mice (Fig. 1c). A new imprinted locus on mouse chromosome 9, corresponding to that of Ras-GRF, has been identified¹³. Our data on Ras-GRF heterozygotes confirmed the reported paternal allele-specific expression (data not shown).

The biological significance of this phenomenon, however, is unknown.

Histological analysis was carried out on adult brains of wild-type and Ras-GRF mutant mice. Nissl-stained coronal sections of mutant mice did not show morphological abnormalities (Fig. 2a, b), despite the fact that Ras-GRF is widely expressed in many CNS structures, including hippocampus, cerebral cortex and thalamus, as shown by *in situ* hybridization analysis of wild-type animals (Fig. 2c). No expression of Ras-GRF messenger RNA was detected in brain sections derived from Ras-GRF^{-/-} mice (Fig. 2d). To study specific subpopulations of neurons in more detail, we used as markers the calcium-binding proteins calbindin-D28K, parvalbumin and calretinin, which are thought to have roles in buffering intracellular calcium and are expressed in different subgroups of neurons¹⁴. Staining patterns were identical in Ras-GRF^{-/-} and wild-type mice, indicating that these calcium-binding proteins were expressed at normal levels (data not shown). Based on the observed behavioural phenotype (see below), we analysed the amygdala in more detail (Fig. 2e, f). This structure also contains high levels of Ras-GRF mRNA and has a normal appearance in the mutants, based on Nissl staining (not shown) and calbindin immunoreactivity. No signs of neuronal atrophy were detected at high magnification (Fig. 2f, inset). In conclusion, our histological analysis did not reveal any major morphological defects in Ras-GRF mutant mice.

Because Ras-GRF-dependent signalling may have a role in synaptic transmission and plasticity, Ras-GRF^{-/-} mice were subjected to behavioural tests. The two-way avoidance test is a measure for both conditioned learning and emotional response to aversive (noxious) stimuli¹⁵. In this test, mice are placed in a two-chamber box and taught to avoid a signalled electric shock (unconditioned stimulus) by running into the opposite compartment. The shock is preceded by a warning light (conditioned stimulus). The role of the amygdala has been clearly demonstrated for this test, as for other emotional learning tests¹⁶. As shown in Fig. 3a, wild-type mice gradually learned to avoid the electric shock with a 40% success rate by day 5 of the experiment. The performance of Ras-GRF mutant mice was much worse, showing a less than 10% success rate on the same day ($P < 0.0001$). Dose-response curves using increasing shock

Figure 1 Generation of a targeted mutation in the mouse Ras-GRF gene. **a**, protein structure, genomic structure and targeting strategy. Location of the 2 pleckstrin homology (PH) domains, the CDC24-like domain and CDC25-like domain are indicated on the structure of p140^{Ras-GRF}. Five exons (designated 1-5) encoding amino-acid sequences N-terminal to the CDC25-like catalytic domain were mapped and sequenced in the murine wild-type Ras-GRF gene and are indicated as vertical bars. A 4-kb region of Ras-GRF containing exons 3-5 was replaced by the PGK promoter-driven neomycin cassette. This caused an increase of 2 kb of a diagnostic *Bam*HI fragment. Two different recombinant clones were used to generate mice carrying the Ras-GRF mutation which were subsequently used for the behavioural tests. Restriction sites: B, *Bam*HI; N, *Not*I; Xb, *Xba*I; Xh, *Xho*I. **b**, Characterization of new polyclonal antibodies against the N-terminal domain of Ras-GRF. Lysates of NIH3T3 cells ectopically expressing Ras-GRF were immunoprecipitated with preimmune serum (lane 1), anti Ras-GRF C-20 antibodies (Santa Cruz Biotech.) (lane 2), affinity-purified anti-N-terminal antibodies in the absence (lanes 3, 4) or presence of the purified PHP antigen (lane 5). After SDS-PAGE, blots were probed with affinity-purified N-terminal (lanes 1, 2, 3) or with C-20 (lanes 4, 5) antibodies. The specific 140K band is indicated. **c**, Biochemical analysis of Ras-GRF mutant mice. A litter of 1-month-old animals derived from a cross between two heterozygotes was first genotyped using polymerase chain reaction (PCR): 400 bp DNA fragment, wild-type allele; 800 bp DNA fragment, mutant allele. Brain extracts were prepared and subjected to western blot analysis with either anti-N-terminal or anti-C-terminal Ras-GRF-specific antisera. Positions of the 140K wild-type protein and of the 100K molecular marker are indicated.



intensities did not show differences between groups, ruling out a possible difference in shock sensitivity (data not shown). Other parameters such as pre-session activity were found to be normal ($P > 0.5$), indicating that the motor activity of both groups in the absence of conditioned stimulus was comparable (not shown). In addition, we have also tested a second Ras-GRF line derived from an independent ES clone targeted in the same way as above. This mutant strain also gave statistically significant impairment in avoidance learning (data not shown).

The one-trial inhibitory avoidance test makes use of the natural tendency of mice to move from an illuminated into a dark compartment¹⁷. Once the animal is in the dark compartment, it receives an electric shock. One single trial is normally sufficient for learning the task, which is to avoid the dark compartment during the probing trial (0.5 h to measure learning and short-term memory or 24 h to measure long-term memory). Note that this test requires no motor activity to manifest learning, in contrast to the two-way avoidance test. Both wild-type and Ras-GRF mutants clearly learn to avoid the dark compartment (Fig. 3b). The step-through latency time at 0.5 h increased in comparison to untrained mice ($P < 0.0001$), but no differences were detected between the groups ($P > 0.1$). In contrast, at 24 h, although wild-type mice persistently avoided the dark chamber, mutant mice seemed to have largely forgotten the task because they ran into the compartment with a latency intermediate between that of 0.5 h and untrained mice ($P < 0.0001$ between the two groups, at 24 h).

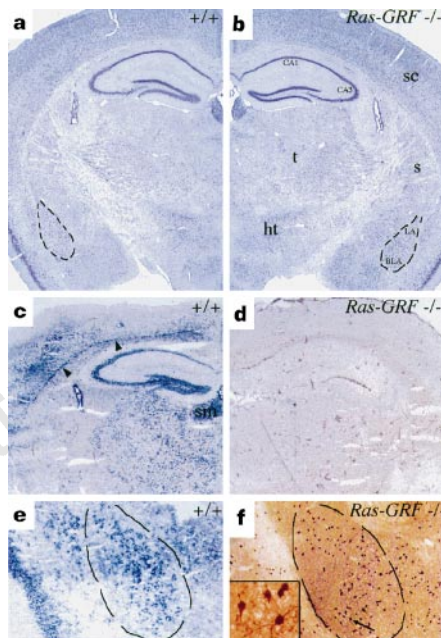


Figure 2 Ras-GRF mutant mice do not show gross morphological abnormalities in the brain. **a, b**, Coronal sections of wild-type and Ras-GRF mutant adult brains, stained with cresyl violet (Nissl). Note the normal appearance of the brain structures. sc, Somatosensory cortex; t, thalamus; s, striatum; ht, hypothalamus; LA, lateral nucleus of the amygdala; BLA, basolateral nucleus of the amygdala (stippled line). **c**, expression pattern of Ras-GRF mRNA by *in situ* hybridization. Note high expression levels in hippocampal structures and in the stria medullaris (sm), wide expression in the thalamus and in different cortical layers with more intense staining in a subpopulation of neurons located in layer VI (arrowheads). **d**, No expression of Ras-GRF was detected in the mutant section corresponding to **c**. **e**, Expression of Ras-GRF mRNA in the wild-type amygdala. **f**, Normal appearance of the lateral and basolateral nuclei of the amygdala of Ras-GRF mutant mice stained with calbindin-D28K. Inset in **f** is a higher magnification (neurons indicated with an arrow). Magnification: **a-d**, X25; **e, f**, X200; inset, X1,000.

One-session fear conditioning can also be studied using a technique in which the foot shock used as an unconditioned stimulus is of higher intensity than in the shuttle-box, but only applied during a short period. When exposed to conditioned stimulus again, conditioned animals show an immobility ('freezing') reaction during which the animals refrain from all but respiratory movements. Freezing responses can be triggered with two different types of conditioned stimulus, each involving different brain structures¹⁸. In contextual conditioning, the conditioned stimulus is represented by the environment in which the unconditioned stimulus is delivered. This type of conditioning appears to depend on both hippocampal and amygdalar functions. In cued conditioning, the conditioned stimulus is a tone, and this type of conditioning is disrupted by lesions of the amygdala but not of the hippocampus. Mice were conditioned to tone and context during the same trial session and then tested separately, 0.5 and 24 h later. For measuring contextual learning, mice were placed in the same box where the training occurred and freezing was monitored for 2 min (Fig. 3c). At 0.5 h after conditioning, both wild-type and mutant mice showed the same freezing response, without significant difference ($P > 0.1$) but statistically higher than untrained mice ($P < 0.0001$). However, at 24 h, although wild-type mice still retained a strong freezing response, Ras-GRF mutants showed a dramatically reduced response ($P < 0.0001$). For measuring cued conditioning, mice were placed in a neutral cage for 1 min to minimize the contextual response, before delivering a continuous

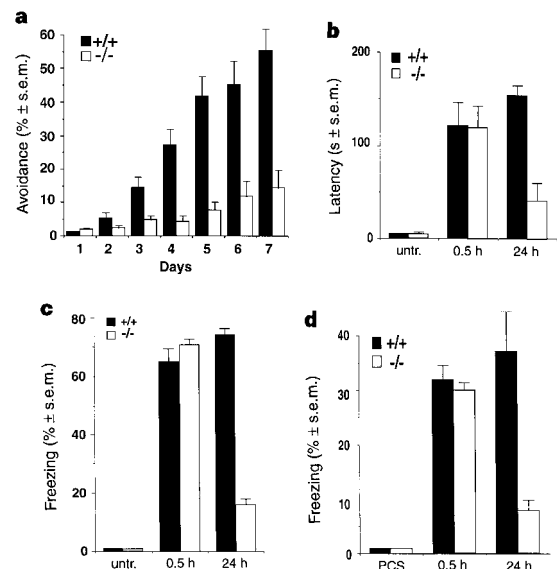


Figure 3 Impaired memory consolidation in Ras-GRF mutant mice during fear conditioning tests. **a**, Two-way avoidance learning test. Mice (wild-type, $n = 24$; mutants, $n = 26$) were trained for 7 days, 80 trials a day. Percentage of correct responses (avoidance of the shock) is shown. **b**, One-trial inhibitory avoidance test. Different groups of mice were tested for step-through latencies into the dark compartment: untr., untrained mice (wild-type $n = 18$; mutant $n = 18$), 0.5 h (wild-type $n = 8$; mutant $n = 8$) or 24 h (wild-type $n = 10$; mutant $n = 10$) after training. Mean step-through latencies expressed in $s \pm s.e.m.$ are indicated for both groups. **c**, Contextual fear conditioning test. Wild-type ($n = 12$) and mutant mice ($n = 10$) were tested before training (untr.), 0.5 h and 24 h after training for freezing (2 min) in the same training box. **d**, Cued fear conditioning test. The same mice used for contextual conditioning were subsequently tested 0.5 h and 24 h after training for freezing in the presence of a sound continuous for 1 min in a neutral cage, different from the training box, to minimize context-dependent freezing response. PCS is the pre-conditioned stimulus phase of 1 min. Cumulative percentages of freezing $\pm s.e.m.$ are indicated.

tone for 1 min (Fig. 3d). As observed for the one-trial inhibitory avoidance test and contextual fear conditioning, acquisition and short retention of the task appeared normal in the Ras-GRF^{-/-} mice, whereas long-term memory was significantly diminished ($P < 0.0001$).

In conclusion, all these emotional learning paradigms clearly demonstrate that Ras-GRF^{-/-} mice are severely impaired at the level of memory consolidation, rather than in the learning process itself.

In contrast to amygdala-mediated fear conditioning, spatial learning mainly depends on the function of the hippocampus¹⁹. To monitor spatial learning in rodents, animals were subjected to the Morris swimming navigation test²⁰. In this test, the animal is placed in a pool and learns to find a submerged platform using visual cues outside the maze. After the intensive acquisition phase lasting 3 days, the platform is moved to the opposite position to test first for spatial learning of the former position (first trial, day 4) and subsequently for suppression of the old spatial information and

reprogramming towards the new goal position (day 4 and 5). Clear learning curves were observed for the mutants ($P < 0.001$), with no differences in escape latency between mutant and control mice ($P > 0.1$) (Fig. 4a). Further evidence for normal spatial learning in both groups came from the retention test (Fig. 4b). During the first trial after the platform was moved to the opposite position, both groups showed preferential searching in the old goal quadrant relative to the others ($P < 0.0001$) but no differences between genotypes were seen ($P > 0.1$), indicated as the number of annulus quadrant crossings.

The radial-arm maze is another sensitive assay for hippocampal function. The advantage over the swimming test is that much less demanding motor activity is required to perform the task and different strategies can be used by the animal to explore the environment. Animals with hippocampal lesions are clearly impaired when tested on this task²¹. The performance of wild-type and mutant Ras-GRF mice was compared in a radial 8-arm maze with cues outside the maze. The procedure used allows the animals to visit each arm, searching for the food reward at any time. Variance analysis on the number of correct arm choices demonstrated that both groups of mice made significant progress in learning performance on the radial maze over the ten training sessions ($P < 0.0001$) (Fig. 4c). Both groups reached roughly the same level of performance and no difference between groups was apparent ($P > 0.1$). In addition, no differences in the strategy used to explore the arm were seen between the two groups (not shown). These data indicate that Ras-GRF mutants do not show major deficits in standard tests of hippocampal function.

To analyse several aspects of synaptic physiology and plasticity we performed electrophysiological experiments in the CA1 region of hippocampus and in the basolateral amygdala. In particular, we tested long-term potentiation (LTP), which is believed to be an important physiological event underlying learning and memory formation²². In CA1, stimulation of the Schaffer collateral pathway at 100 Hz in slices from Ras-GRF mutant and wild-type mice induced LTP of the synaptic response (Fig. 5a). This potentiation was not significantly different in slices from mutant mice ($162 \pm 14\%$; $n = 11$ slices, 5 animals) compared to slices from wild-type mice ($158 \pm 13.8\%$; $n = 13$ slices, 6 animals). The induction of LTP under these conditions was blocked when tetani were delivered in the presence of the NMDA receptor antagonist D-AP5 ($50 \mu\text{M}$) (data not shown). Extracellular field potential responses were recorded in the basolateral amygdala, which is known to support plasticity *in vitro*²³. Delivery of 3 trains of stimulation of 10-burst theta frequency stimulation produced significant LTP (Fig. 5b, c) in $+/+$ controls ($134 \pm 12\%$ of baseline; $n = 8$ mice, 16 slices), whereas $-/-$ mice showed no such enhancement ($104 \pm 3\%$ of baseline; $n = 7$ mice, 11 slices, $P < 0.001$). Although long-lasting plasticity was absent in $-/-$ mice, there were no differences ($P = 0.78$) between the two groups in the first minute after tetanus ($-/-$, $122 \pm 3\%$; $+/+$, $124 \pm 4\%$). When theta-burst tetanus was delivered to Schaffer collaterals in the CA1 region of hippocampal slices (taken from the same mice as the amygdala slices) LTP in $-/-$ mice ($130 \pm 13\%$ of baseline, $n = 6$ mice) was not different from LTP in $+/+$ mice ($121 \pm 10\%$ of baseline, $n = 4$ mice, $P > 0.5$, Fig. 5b). Analysis of baseline synaptic response properties revealed that Ras-GRF^{-/-} mice showed larger field excitatory post-synaptic potentials (EPSPs) in amygdala than $+/+$ mice (mean across intensities for $-/-$ mice was $0.94 \pm 0.04 \text{ V s}^{-1}$; $n = 9$ mice, 15 slices; for $+/+$ mice, 0.54 ± 0.04 , $n = 14$ mice, 25 slices) and in CA1 of hippocampus (mean across intensities for $-/-$ mice was $2.55 \pm 0.14 \text{ V s}^{-1}$; $n = 8$ mice, 10 slices; for $+/+$ mice, 1.44 ± 0.12 ; $n = 11$ mice, 14 slices), across a range of stimulus intensities (Fig. 5e). Analysis of variance indicates that the differences were significant in both structures ($P < 0.0001$). In the hippocampus, it is possible to use the amplitude of the presynaptic fibre volley to estimate the strength of afferent inputs, and thus

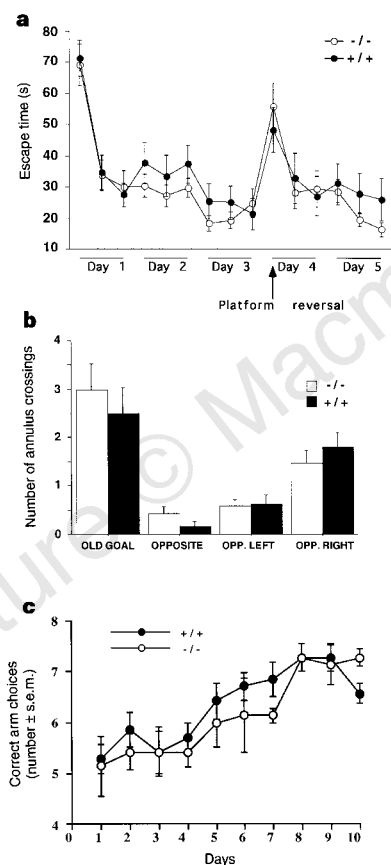


Figure 4 Hippocampal-dependent behaviour appears to be normal in Ras-GRF mutant mice. **a**, Spatial learning test. Wild-type ($n = 24$) and mutant mice ($n = 24$) were trained for 3 consecutive days (6 trials per day) with the submerged platform (acquisition phase) which was followed by 2 days of reversal phase, with the platform at the opposite position in the pool. Escape latency is expressed in seconds required to find the platform. **b**, Number of annulus crossings of the quadrants at the probing trial (day 4, trial 1). The value is indicated for all four quadrants: old goal, old training quadrant; opposite, opposite quadrant to the old goal position; opp. left, adjacent left quadrant to the old goal; opp. right, adjacent right quadrant to the old goal. **c**, Radial-maze test. Wild-type ($n = 14$) and mutant ($n = 14$) mice were trained for 10 consecutive days in an eight-arm radial maze. Learning performance is expressed in terms of the mean number of correct arm choices on each trial. The number observed at day 1 was taken as the starting point (5.28 ± 0.28 for wild-type; 5.14 ± 0.59 for mutants).

compare the synaptic input/output values more directly. Measurements of the ratio of the EPSP slope to the fibre volley amplitude (Fig. 5f) also indicated significant differences ($P < 0.0001$) between $+/+$ mice (mean ratio = 2.27 ± 0.23 ; $n = 11$ mice, 13 slices) and $Ras-GRF^{-/-}$ mice (mean ratio = 3.96 ± 0.27 ; $n = 8$ mice, 10 slices). Finally, we examined paired-pulse facilitation (Fig. 5d), a form of short-lasting plasticity that depends on presynaptic mechanisms²⁴. In hippocampus, wild-type mice show paired-pulse facilitation values (1.38 ± 0.07) that were statistically indistinguishable from $Ras-GRF$ mutant slices (1.30 ± 0.02 , $P > 0.5$). The same was observed for amygdala slices (for $+/+$, 1.27 ± 0.05 ; for $-/-$, 1.19 ± 0.03 , $P > 0.1$), suggesting that $Ras-GRF$ is not absolutely necessary for this type of plasticity.

We have shown that $Ras-GRF$ mutant mice have an impairment in the process of memory consolidation during fear-related behavioural tasks and electrophysiological impairments in the amygdala, a critical part of the neural circuitry involved in emotional responses. The role of the amygdala in regulation of the behavioural response to external and noxious stimuli has been clearly demonstrated^{25,26}. In rats, lesions in the amygdala dramatically affect acquisition of both cued and contextual fear conditioning, whereas specific lesions at the level of the hippocampus affect only contextual fear conditioning¹⁸. In contrast, $Ras-GRF$ mutant mice do not show deficits in the learning process itself, implying that $Ras-GRF$ signalling seems to be specifically involved in the consolidation of long-term memory.

Although $Ras-GRF$ is highly expressed in the CA1 region of the rodent hippocampus, we did not detect, using several standard protocols, any major abnormalities in hippocampal synaptic plasticity or hippocampus-dependent forms of learning. We cannot infer that hippocampal functions are completely normal, as there are differences in some aspects of synaptic transmission, and there may be changes in other forms of plasticity. In any case, $Ras-GRF$ does not seem absolutely required for spatial learning. Alternatively, some compensatory events might have occurred in the $Ras-GRF$ mutants to mask the hippocampal phenotype.

Currently there is little information on the mechanisms by which $Ras-GRF$ might be involved in processes leading to changes in synaptic transmission and LTP. We favour the idea that $Ras-GRF$ signalling is directly involved in synaptic plasticity, although an indirect effect on the activity of certain types of neurons in the amygdala cannot formally be excluded. The differential effects on synaptic plasticity between amygdala and hippocampus may reflect the ability of $Ras-GRF$ to couple with distinct signal transduction mechanisms that predominate in each of these structures. LTP in the CA1 region of the hippocampus requires calcium influx via the NMDA receptor, in contrast to at least some important pathways in the basolateral amygdala in which LTP is NMDA receptor-independent²⁷. Moreover, muscarinic receptors are highly expressed in the basolateral amygdala and muscarinic antagonists block LTP in this structure²⁸. Because muscarinic receptors activate $Ras-GRF$, producing an increase in its phosphorylation⁶, this pathway may be physiologically relevant to amygdala function. Thus $Ras-GRF$ may be important in synapses where metabotropic receptors dominate synaptic plasticity and less important in those regulated by NMDA receptors.

Knockout experiments have demonstrated a role in long-term plasticity and behaviour for a number of protein kinase cascades²⁹. Our finding that the $Ras/Raf/MAP$ kinases pathway controlled by $Ras-GRF$ is also involved in such processes strengthens the notion that multiple signalling events are simultaneously required for the generation of long-lasting synaptic changes, leading to consolidation of learning and memory processes. □

Methods

Generation of targeted mice. To construct the $Ras-GRF$ targeting vector, a 1.3 kb *XhoI*–*NotI* and 5.6 kb *XbaI*–*XbaI* DNA fragments of cloned 129 strain $Ras-GRF$ genomic DNA were used. After homologous recombination, a neomycin-resistance cassette was inserted while deleting 4 kb of the $Ras-GRF$ locus, including exons 3–5 coding for the 5' portion of the CDC25-like catalytic domain (nucleotides 2,982–3,260 of the published cDNA sequence)³. Germline-transmitting chimaeras from two recombinant cell lines were

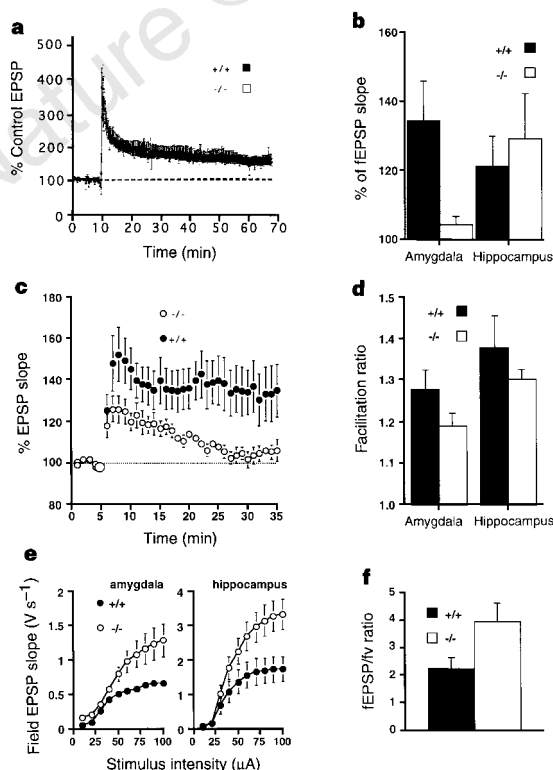


Figure 5 Impaired synaptic plasticity in the amygdala of $Ras-GRF^{-/-}$ mice. **a**, LTP in the hippocampus is unaffected by the $Ras-GRF$ mutation following 2 trains of 100 stimuli each at 100 Hz. **b**, In both control and mutant hippocampal slices, theta-burst stimulation produces significant potentiation. **b**, **c** In $+/+$ amygdala slices, theta-burst stimulation is also an extremely effective protocol for LTP induction, but amygdala slices taken from $-/-$ mice do not demonstrate significant potentiation 30 min after tetanus. **d**, Paired pulse facilitation is not different between wild-type and $Ras-GRF^{-/-}$ mice, in either the hippocampus or the amygdala. The indicated data are the average across the 30–100 μA range of stimulus intensities, at 30-ms interpulse intervals. **e**, Synaptic responses are significantly elevated in both amygdala slices (left) and hippocampal slices (right) taken from $Ras-GRF^{-/-}$ mice, compared to $+/+$ controls. **f**, After controlling for variability in afferent input strength by measuring the ratio of the field EPSP slope to the fibre volley amplitude in hippocampal slices, the difference between $+/+$ and $-/-$ mice was still significant.

obtained by standard injection into C57BL/6 blastocysts, and the mutation was crossed into either 129/Sv or C57BL/6 genetic backgrounds.

Biochemistry. The Ras-GRF N-terminal fragment (PHP) corresponding to residues 1–149 of the published sequence³ was used to raise specific antibodies. Total adult brain protein lysates were subjected to western blot analysis using polyclonal antibodies directed either against the N-terminal or the C-terminal portion of p140^{Ras-GRF}. Blots were developed using the ECL method.

Histology. For immunohistochemistry, 40- μ m coronal cryosections were prepared and further processed using standard techniques. For *in situ* hybridization analysis, 16- μ m sections were prepared and processed following the manufacturer's protocol for digoxigenin-labelled oligonucleotide probes (Boehringer Mannheim). The DNA fragment used as a probe corresponds to 3' sequences of the published cDNA sequence of mouse Ras-GRF (nucleotides 1,915–4,174).

Behavioural tests. The mice used for all the behavioural tests were littermates of 9–16 weeks of age, kept on a 1:1 mixed genetic background between 129/Sv and C57BL/6. All the behavioural tests were performed as previously described^{15,17,20,21,30}. In brief, for the two-way avoidance test, mice were placed in sound-proof shuttle-boxes (Campden Instruments) operated by a computer. After 2 min during which the mice were left undisturbed (pre-session), a conditioning light stimulus lasting 5 s was delivered and followed by a 10 s electric shock of 0.15 mA. Intertrial interval varied between 5 and 15 s. The animals underwent 80 trials a day for 7 days.

For the one-trial inhibitory avoidance test, mice were trained on an apparatus in which a straight alley was divided into two compartments. The smaller compartment was made of white Plexiglas. The larger one was made of black Plexiglas and was equipped with a removable cover of the same material to allow the compartment to be in darkness. A tensor lamp illuminated the small compartment. The floor of the larger compartment consisted of two oblique stainless steel plates folded at the bottom through which a constant current could be delivered. On the training day each mouse was placed in the lit compartment, facing away from the dark compartment. When the mouse had stepped with all four paws into the dark side, the door was closed, the step-through latency was recorded, and two foot shocks (0.4 mA, 50 Hz, 2 s) were delivered with an interval of 5 s. The maximum initial step-through latency allowed as a criterion for the animals entering the trial was 15 s. The mouse was then removed from the apparatus and returned to its home cage. Retention was tested 0.5 or 24 h later following a similar procedure, except that no shock was administered. A maximum step-through latency of 180 s was allowed in the test session.

For both contextual and cued fear conditioning, mice were trained within the same session, with the following protocol: the pretrial time of 1 min in the conditioning box (the same used for shuttle box) was followed by 15 s tone (conditioned stimulus, 3,000 Hz, 80 dB). During the last 5 s of tone a foot shock of 0.75 mA was delivered and after 15 s the procedure was repeated 5 times. During the training period, acquisition of the freezing response was monitored and no differences were found between the two groups. The freezing response to conditioned stimulus was monitored 0.5 h and 24 h after training for both tests: for contextual conditioning, mice were monitored for freezing for 2 min in the box used for training. For cued conditioning, mice were placed in a new, neutral cage, and freezing was monitored for 1 min in the absence of sound (pre-conditioned stimulus freezing) and for 1 min in the presence of a continuous sound (conditioned stimulus freezing). In both tests freezing was scored every 5 s.

The Morris navigation test was carried out in an open-field water maze of 1.5 m in diameter and filled with opaque water at the temperature of $25 \pm 1^\circ\text{C}$, located in a laboratory that contained prominent extra-maze cues. A hidden 15-cm-diameter platform was used. Trials lasted a maximum of 120 s. Spatial training consisted of 18 trials (6 per day) during which the platform was left in the same position. After 3 days of learning, the platform was moved to the opposite position and reversal learning was monitored for 2 additional days (6 trials per day).

For the radial maze test the apparatus was a grey plastic maze with eight identical arms radiating from an octagonal starting platform (perimeter, 7×8 cm). On each training trial a 20-mg food pellet was placed at the end of each arm and the animal was placed facing a randomly selected direction on the central platform. Animals received one trial per day; each daily trial terminated

when eight choices were made or 15 min had elapsed. An arm choice was defined as placement of all paws on a maze arm. An error was committed when an animal enters a previously visited arm.

Electrophysiology. Slices of amygdala and hippocampus were prepared using standard methods and media. Brains were removed to ice-cold artificial cerebrospinal fluid (ACSF, 119 mM NaCl, 2.5 mM KCl, 1.3 mM MgSO₄, 1.0 mM NaH₂PO₄, 26.2 mM NaHCO₃, 2.5 mM CaCl₂ and 11 mM glucose), cut on a Vibratome or a gravity tissue chopper at 400 μ m, and maintained in a submersion-type chamber at 28–32 $^\circ\text{C}$. Extracellular field potentials were recorded using glass pipettes filled with 1 M NaCl or carbon-fibre electrodes placed in the basolateral amygdala or in stratum radiatum of hippocampal CA1. CA1 responses were evoked by stimulation of the Schaffer collaterals using bipolar or monopolar stainless steel electrodes, and responses in the basolateral amygdala were elicited by stimulation of the lateral amygdala with monopolar stainless steel electrodes.

Received 18 April; accepted 15 August 1997.

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Acknowledgements. We thank F. Casagrande for her help with ES cell work, A. Plück, K. Brennan and M. Lemaître for generating germline chimaeras from the second independent ES cell clone, K.-P. Giese and A. Silva for providing their Ras-GRF mouse mutant strain before publication, R. Morris, R. Zippel, E. Martegani, L. Alberghina, A. Oliverio and P. Orban for critically reading the manuscript and F. Peverali for helping with artwork. R.B. was supported by a long-term Human Frontier Science Program Organization (HFPO) postdoctoral fellowship, L.M. by a long-term EMBO fellowship, S.G.N.G. and C.H. by the Wellcome Trust, and A.R. by BBSRC. The work was partially supported by the Italian Association for Cancer Research (AIRC), by Progetto Finalizzato ACRO of the Italian National Research Council (CNR) and by CEE (to E.S.), by the Swiss National Science Foundation (to H.-P.L. and D.P.W.), by HFPO (to H.-P.L. and S.G.N.G.) and by MRC (to P.F.C.).

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Short-range control of cell differentiation in the *Arabidopsis* root meristem

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Meristems are distinctive regions of plants that have capacity for continuous growth. Their developmental activity generates the majority of plant organs¹. It is currently unknown how cell division and cell differentiation are orchestrated in meristems, although genetic studies have demonstrated the relevance of a proper balance between the two processes^{2–6}. Root meristems contain a distinct central region of mitotically inactive cells, the quiescent centre⁷, the function of which has remained elusive until now. Here we present laser ablation and genetic data that show that in *Arabidopsis thaliana* the quiescent centre inhibits differentiation of surrounding cells. Differentiation regulation occurs

within the range of a single cell, in a manner strikingly similar to examples in animal development, such as during delamination of *Drosophila* neuroblasts⁸. Our data indicate that pattern formation in the root meristem is controlled by a balance between short-range signals inhibiting differentiation and signals that reinforce cell fate decisions⁹.

Plants develop and maintain groups of stem cells called meristems. Meristems control the development of plant organs through balanced cell proliferation and differentiation. In the *Arabidopsis thaliana* root, files of all different cell types originate from the meristem (Fig. 1a). Each cell file begins in an initial cell (low colour intensity), which performs stem-cell like divisions to generate a new initial and a daughter cell. The daughters undergo a small number of divisions and differentiate further¹⁰. There is a strong correlation between cell type and embryonic lineage, but cell fate requires continuous positional signalling⁹. The initial cells surround the four mitotically inactive cells of the quiescent centre (QC; grey in Fig. 1a)⁷. We have exploited the well-defined location and the small size of the QC in the *Arabidopsis* root meristem to analyse its function through laser ablation and genetic studies. The QC has been proposed to function as a stem cell reservoir to replace damaged cells¹¹ or as an 'organizer' of cellular pattern^{12,13}. Laser ablation of initial cells has shown that many cells can replace the ablated cells⁹ and the QC therefore is not a unique stem cell population. Follow-

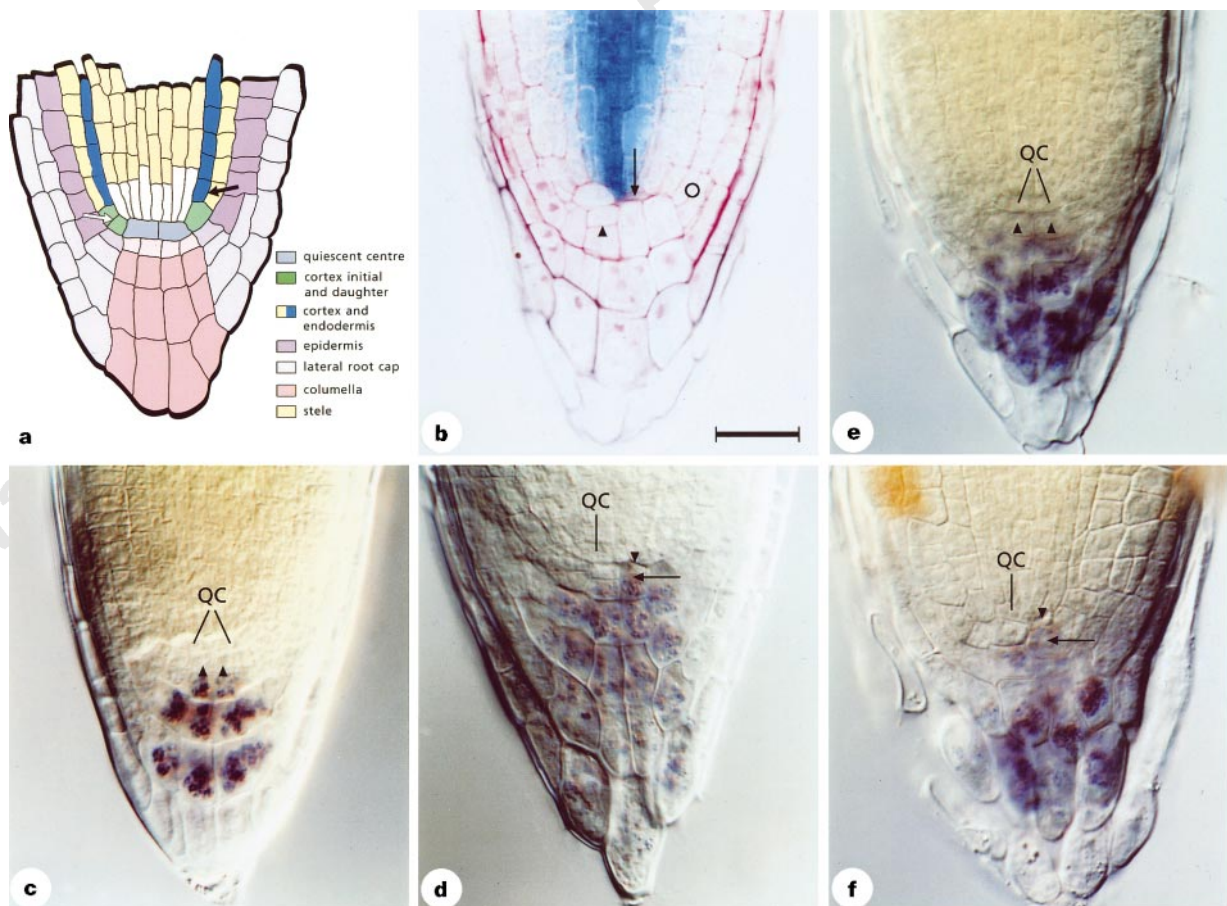


Figure 1 QC cells inhibit differentiation of contacting columella initials. **a**, Schematic representation of the cell types present in the *Arabidopsis thaliana* root meristem. The QC contacts initials (low colour intensity) of all cell types. White arrow: cortical initial divides into a daughter cell. Black arrow: cortical daughter forms cortex and endodermis. **b**, Ablation of 1 QC cell (arrow) results in cessation of division of the contacting columella initial (94.8%, $n = 77$). The columella initial underlying the intact QC cell still divides (arrowhead). Circle, division of epidermal initial generating lateral root cap (89.6%, $n = 48$). **c**, Starch

granules in wild-type *Arabidopsis* columella cells. The columella initials (arrowheads) lack starch. **d**, Progression of differentiation of contacting columella initials (arrow) after QC ablation (arrowhead). **e**, Post-embryonic cell division mutants have a wild-type pattern of starch granule distribution (arrowheads: columella initials). **f**, One QC cell ablation (arrowhead) in post-embryonic cell division mutants results in progression of differentiation of contacting columella initials (arrow; Table 1). Scale bar, 25 μm.

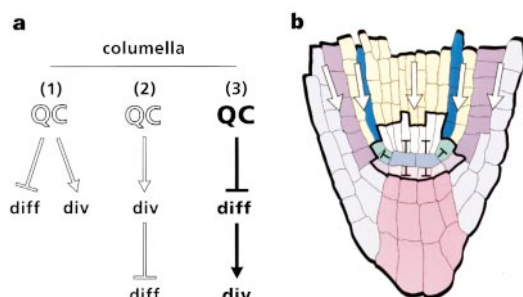


Figure 2 Models for the regulation of cell differentiation and cell division of columella initials by the QC (**a**), and a generalized model for the control of differentiation of cells in the root meristem (**b**). **a**, The QC is either involved in regulating both differentiation (diff.) and division (div.) independently (1), in promoting division which prevents differentiation (2) or in inhibiting differentiation which allows division (3). Our data support model 3. **b**, We suggest a simple model where signals guiding position-dependent differentiation originate from more mature cells and reach the initial⁹ (arrows). Initial cells differentiation is inhibited through their contact or close proximity to the QC (inhibitory signals). Upon division of the initials, daughter cells no longer contact the QC and no longer receive the QC signals. They now respond to signals from their more mature daughters that guide further differentiation into the corresponding cell type.

Table 1 Columella initials differentiate upon QC ablation

	Initials	Columella Daughters
wt control	0%	100% (n = 40)
wt QC ablation	100%	100% (n = 33)
c6044 control	0%	100% (n = 30)
c6044 QC ablation	100%	100% (n = 19)
c4761 control	14.1%	100% (n = 106)
c4761 QC ablation	100%	100% (n = 17)
c4912	6.7%	100% (n = 15)
c4976	17.2%	100% (n = 29)

Accumulation of starch granules in columella cells. In control plants (both wild-type and 4 mutants without post-embryonic cell division) starch is present in columella daughters and more basal columella cells but absent in initials. Upon QC ablation, initial cells differentiate as indicated by starch accumulation. c4761, c4912 and c4976 express starch at low frequency. Therefore we cannot exclude minor contributions of cell division to differentiation status.

ing complete QC ablation, the QC is rapidly restored by cells of the stele⁹, thus preventing analysis of its function. We therefore analysed the effects of ablating only one or two QC cells, which results in slower replacement (see Methods).

Ablation of one QC cell results in cessation of cell division of columella initials (dull pink cells in Fig. 1a) which are in direct contact with the ablated cell (Fig. 1b). This effect is specific, as non-QC cell ablation next to columella initials (for example, columella daughters (pink in Fig. 1a)) does not result in mitotic arrest. After ablation of one QC cell, the columella initials contacting the remaining intact QC cells still perform their normal asymmetric division (Fig. 1b, arrowhead). This demonstrates that the influence of QC cells does not extend to non-contacting columella cells. We concluded that individual QC cells promote columella cell division within a single cell range.

We followed the fate of the mitotically arrested columella cells through the analysis of a columella-specific marker. Mature columella cells contain starch granules that are not found in initial cells (Fig. 1c; Table 1). After QC ablation, starch granules appear in the underlying elongated initials (Fig. 1d, arrow; Table 1). This shows that the differentiation state of columella initials progresses upon QC ablation. Therefore, the QC can arrest differentiation thus allowing division, can promote division thus preventing differentiation, or can regulate both (Fig. 2a).

To test whether cell division is required to prevent differentiation of columella initials, we analysed the QC activity in mutants lacking post-embryonic root meristematic cell divisions. We characterized four independent *Arabidopsis* mutants (c4761, c4912, c4976 and c6044) defining at least three genetic loci. These mutants have a wild-type cellular architecture of the root meristem but lack post-embryonic cell divisions. Root cell number counts¹⁴ reveal that the embryonic cell number did not increase in the mutants (not shown). As in wild type, starch is absent in the columella initial cells but is present in the daughters (Fig. 1e; Table 1), showing that the initial status is independent of cell division. QC ablation in these

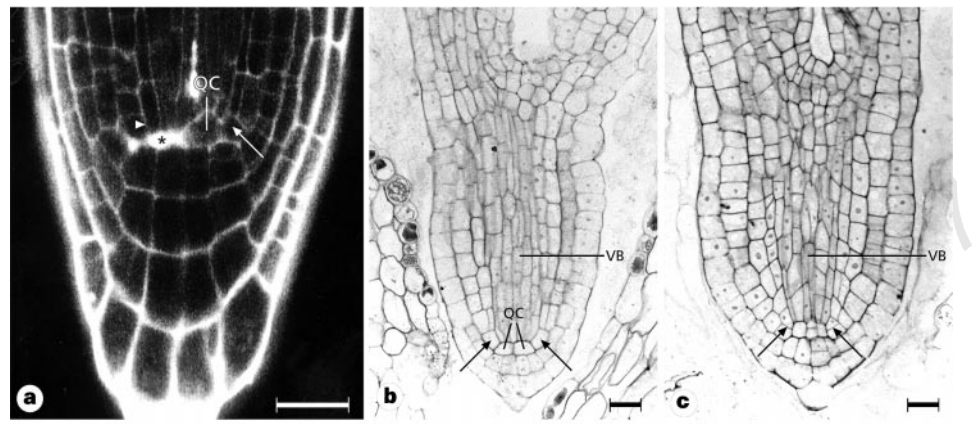
mutants results in starch accumulation in columella initials as observed in wild-type roots (Fig. 1f, arrow; Table 1). The QC thus controls the differentiation state of columella initials in the absence of cell division. These results show that columella cell division is not a prerequisite for inhibition of differentiation (Fig. 2, model 2).

To determine whether the QC cells also control the differentiation of other initial cells, we examined the differentiation of cortical initials (green; Fig. 1a) after QC ablation. Cortical initials normally generate cortical daughters. These generate an inner layer of endodermal and an outer layer of cortical cells (blue and yellow, respectively, Fig. 1a) by an asymmetric division of daughters but not initials (Fig. 1a, arrows; see Methods). When one or two QC cells are ablated, directly contacting cortical initials behave as cortical daughters and divide asymmetrically into cortical and endodermal cells (Fig. 3a, arrowhead). Cortical initials contacting intact QC cells divide normally into cortical daughters (Fig. 3a, arrow). Removing the QC thus results in the progression of differentiation of contacting cortical initials. We conclude that the QC inhibits differentiation of contacting cortical initial cells.

Genetic evidence corroborating the effect of the QC on cortical initials comes from mutations in the *HOBBIT* (*HBT*) gene. In *hbt* mutant embryos, the progenitor cell of the QC and columella undergoes aberrant divisions. As a consequence, an aberrant QC is formed and no functional columella is present (Fig. 3c) (V.W. and B.S., manuscript submitted). In these mutants, all cortical cells divide into cortex and endodermis during embryogenesis (Fig. 3c, arrows; compare with Fig. 3b), inferring that without a functional QC the initial status cannot be maintained.

We questioned whether the QC functions primarily as a negative regulator of cell differentiation independent of cell division (Fig. 2a, model 1), or whether it regulates cell division through differentiation (Fig. 2a, model 3). We investigated this by examining cell divisions after QC ablation. In wild-type roots, divisions in the columella initials and the epidermal initials are always in a similar order: columella initials always divide before epidermal initials (100%; n = 56). After ablation of one QC cell, contacting columella initials no longer divide, whereas both epidermal initials contacting the dead QC cell (Fig. 1b, circle) and columella and epidermal initials contacting the intact QC cells (Figs. 1b, 3a) divide normally. Second, we observed new cell walls in cortical initials (see above, Fig. 3a). Third, ³H-thymidine incorporation in QC-ablated roots shows that cells surrounding the QC passed S-phase at rates similar to those in non-ablated roots (not shown). Together, this shows that all surrounding cells except columella cells are still able to divide at rates indistinguishable from wild type. It is noteworthy that in wild type, only the columella daughters lack division whereas in all other cell types daughters undergo further divisions while differentiating. Therefore, it is only in the columella that a progression of the initial stage into the next stage of differentiation infers the cessation of division. We conclude from these experiments that it is most likely that the QC does not directly regulate cell division in the columella but primarily arrests cell differentiation (Fig. 2a, model 3).

Figure 3 The QC arrests differentiation of cortical initials. **a**, Upon ablation of 1 QC cell (asterisk), contacting cortical initials divide in a cortical daughter-specific manner (arrowhead) indicating progression of differentiation ($n = 184$) (arrow: normal cortical initial). **b**, Wild-type torpedo-stage embryo (arrows: cortical initials, VB: vascular bundle). **c**, *hobbit* torpedo-stage embryo lacks a typical QC. Cortical cells have divided into cortex and endodermis (arrows; $n = 104$). Cortical initials are recognized because they are always located next to the vascular bundle (VB; compare Figs 1a and 3b). Scale bars, 25 μm .



In the *Arabidopsis* root meristem, positional signals for proper differentiation appear to derive from more mature cells to guide cell fate of initials of the same cell type⁹. We show here that contact with QC cells keeps cells in an initial-specific, less differentiated state. A balance between these two antagonistic signals could be the mechanistic basis for orchestrating pattern formation in the root meristem (Fig. 2b).

The shoot meristem also contains a central region of cells with reduced division rates¹. Genetic analyses in *Arabidopsis* suggest that this region regulates the rate of either cell division or cell differentiation^{4–6}. *clavata* (*clv*) mutants display overproliferation of the central zone of the shoot meristem^{15,16}. The *CLV1* gene encodes a putative serine-threonine receptor kinase, inferring that receptor–ligand interactions are either required to promote cell differentiation or to inhibit cell division in meristems¹⁷. We show here that in root meristems, central cells control cell differentiation rather than cell division. Furthermore, we show that inhibition of differentiation is within single-cell range and possibly cell-contact dependent. This provides a striking analogy to controls of cell differentiation in *Drosophila*, such as the NOTCH-DELTA receptor–ligand interaction that represses the differentiation of neuroblasts by contact-dependent mutant inhibition⁸. □

Methods

Cell ablation experiments and analyses. Cell ablation experiments and sectioning were done as described^{9,10}. Ablation of the whole QC (4 cells) results in a rapid replacement by the stele (percentage of replacement: 0%, 50.7%, 75% and 82.4% at 1, 2, 3 and 4 days after ablation, respectively). Ablation of 1 or 2 QC cells results in a slower replacement (0%, 10.3%, 43.3% and 46.2% at 1, 2, 3 and 4 days after ablation, respectively). Only roots where replacement had not started were further examined. The effects we observed were specific for the QC because ablation of all vascular initials above 1 QC cell did not result in any defects. This shows that the QC ablation effects are not the result of a longer-range apical–basal-directed signal. Furthermore, the effects we observed are not likely to be a wounding effect because, following QC ablation, we observed specific cell divisions (periclinal division of epidermal initials), whereas columella initials cease division.

To analyse cortical initial divisions in both the wild-type control and QC-ablated plants, transverse sections were made using a confocal laser scanning microscope⁹. Divisions of each individual cortical cell were counted before ablation and 2, 3 or 4 days after ablation. In wild-type control roots (Col-0), these divisions ranged from 3.1% (start of experiment) to 12.0% (end of experiment) ($n = 584$ cells). In Ler and C24, similarly low numbers of cortical divisions were observed (11.6%, $n = 112$; 19.4%, $n = 72$). NO-O was an exceptional ecotype, as the percentage was much higher (86.5%, $n = 96$). In cortical cells not contacting the ablated QC, cell divisions were similar to control plants (1.4% at the start of the experiment to 11.6% at the end; $n = 552$) showing that the ablation has no effect on divisions of non-contacting cortical initials. In cortical initials contacting the ablated cells, a significantly

higher percentage divided (2.2% start to 47.8% end, $\chi^2 P < 0.05$; $n = 184$ using SPSS 6.1).

Starch was visualized in whole roots by incubation for 3 min in 1% Lugol (Merck). Roots were mounted in 75% chloralhydrate (Merck) and examined with Nomarsky optics. After QC ablation, roots were grown for 4 days on agar plates containing 2 $\mu\text{Ci m}^{-1}$ ^3H -thymidine (TRK.418, Amersham).

Analysis of mutants. Mutants lacking post-embryonic cell divisions were isolated in a genetic screen using EMS-mutagenized Col-0 seeds¹⁸. Complementation was observed between *c4912* and *c4761*, between *c4912* and *c6044* and between *c4761* and *c6044*, establishing 3 genetic loci. Isolation and characterization of *hobbit* mutants will be described elsewhere (V.W. and B.S., manuscript submitted). Asymmetric periclinal cortical divisions in wild-type embryos (from heart stage onwards) (2.2%, $n = 44$) were compared to strong *hobbit* (2311: 80%, $n = 30$; 5859: 66.7% $n = 18$; GVII-24/1: 62%, $n = 34$) and a weak *hobbit* allele (*c56*: 86% $n = 22$).

Received 22 May; accepted 22 August 1997.

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Acknowledgements. We thank W. Hage for assistance with the confocal microscope; P. Westers for statistical analysis; D. Smit, F. Kuyter and B. Landman for artwork; F. Kindt, R. Leitho, W. Veenendaal and P. Brouwer for photography; and H. I. McKhann and P. Benfey for useful suggestions on the manuscript. C.v.d.B. was sponsored by a grant from the Dutch Organisation of Life Sciences (SLW).

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Mice lacking factor VII develop normally but suffer fatal perinatal bleeding

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Blood coagulation *in vivo* is initiated by factor VII (FVII) binding to its cellular receptor tissue factor (TF)^{1–4}. FVII is the only known ligand for TF, so it was expected that FVII-deficient embryos would have a similar phenotype to TF-deficient embryos, which have defective vitello-embryonic circulation and die around 9.5 days of gestation^{5–8}. Surprisingly, we find that FVII-deficient (FVII^{–/–}) embryos developed normally. FVII^{–/–} mice succumbed perinatally because of fatal haemorrhaging from normal blood vessels. At embryonic day 9.5, maternal–fetal transfer of FVII was undetectable and survival of embryos did not depend on TF–FVII-initiated fibrin formation. Thus, the TF^{–/–} embryonic lethal and the FVII^{–/–} survival-phenotypes suggest a role for TF during embryogenesis beyond fibrin formation.

The factor VII (FVII) gene was inactivated by deleting the entire coding sequence for the mature FVII protein (Fig. 1a, b). This resulted in an absence of FVII messenger RNA in the liver and of procoagulant activity in the plasma in homozygous FVII-deficient (FVII^{–/–}) neonates (Fig. 1c; Table 1). The FVII^{–/–} embryos developed normally and were represented in a normal mendelian inheritance pattern (see Table S1 in Supplementary information). Vascular development was normal in all 50 FVII^{–/–} embryos at any embryonic age (Fig. 2a, b) and the typical vascular fragility of TF^{–/–} embryos (Fig. 2c) was not seen (see Fig. S1a–f in Supplementary information). Even when deficiency of FVII and TF^{7,8} were compared in mice with the same C57B16/J × 129/SvJ genetic background using large groups of offspring, the 80% embryonic lethality associated with TF deficiency contrasts sharply with the 100% normal FVII^{–/–} embryonic development (Supplementary information Table S1). Thus, apart from a theoretically possible influence from closely linked genes (which appears to be negligible, as indicated by the lack of rNAPc2-induced bleeding in embryos with different genetic backgrounds, see below), differences in genetic background do not appear to account for the observed phenotypes.

In contrast to their normal embryonic development, 70% of the FVII^{–/–} neonates suffered fatal intra-abdominal bleeding within the first 24 h whereas most of the remaining neonates died from intracranial haemorrhage before the age of 24 days (Fig. 2e, f). Microscopic analysis confirmed widespread blood collection in the peritoneal cavity (Supplementary information Fig. S1g, h) or in the subdural cavity (Fig. 2g, h). However, blood vessel development, judged by intact von Willebrand Factor immunoreactive endothelium (Supplementary information Fig. S1i, k) and numerous α -actin-positive smooth muscle cells (Supplementary information Fig. S1j, l) was normal. Fatal perinatal bleeding may also account for the frequent recovery within the first day after birth of fragments

of dead FVII^{–/–} neonates, which were partly consumed by their mothers. Other components of the intrinsic or extrinsic coagulation cascade did not compensate for loss of the essential FVII procoagulant activity (Table 1). Apparently, FVII^{+/–} neonates with their ~10% of adult plasma FVII levels were protected against bleeding. Similar phenotypes were obtained from F₂ offspring derived from two independent FVII-targeted embryonic stem cell clones.

FVII mRNA levels were minimal in the embryonic day 9.5 (E9.5) FVII^{+/+} embryo and yolk sac (consistent with the minimal FVII procoagulant activity in E11.5 embryos; Table 1) but abundant in the liver of postnatal day 2 (P2) neonates and adults (Fig. 1d). FVII-immunoreactivity (FVII-ir) was absent in the E9.5 FVII^{+/+} embryo but became progressively more abundant in the P2 and adult liver (Fig. 3a, d).

In order to examine whether normal development of FVII^{–/–} embryos was dependent on maternal–fetal transfer of FVII, FVII procoagulant activity was determined in plasma from embryos at E11.5, E14.5 and E18.5, sampled by intracardiac puncture to avoid contamination with maternal blood (Table 1). At any embryonic age, FVII activity levels in the plasma of FVII^{–/–} embryos were undetectable ($\leq 0.05\%$ of adult levels) whereas they progressively increased to ~30% of adult levels in wild-type neonates (Table 1). Maternal–fetal transfer of FVII was further investigated by intravenous injection of recombinant human FVIIa (rFVIIa) in pregnant females and measuring rFVIIa within the embryos, using a human FVII-specific ELISA (not crossreacting with murine FVII). At E9.5,

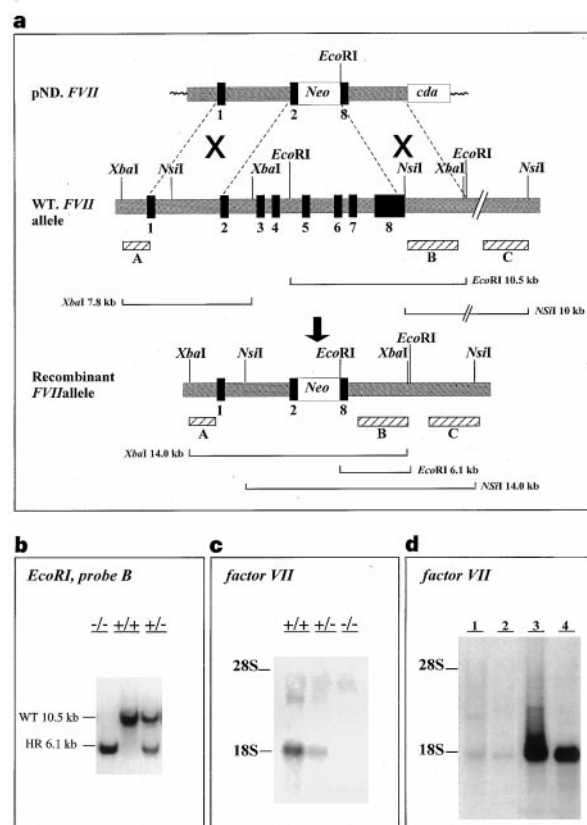


Figure 1 Generation of FVII^{–/–} mice. **a**, Genomic map. The top line represents the targeting vector, pND.FVII, in which the entire coding sequence was replaced with a neomycin resistance (*neo^r*) cassette. The middle and bottom lines show, respectively, the wild-type and recombinant FVII alleles. Darkened blocks, exons; shaded bar, introns. Probes A and C, external; probe B, internal. **b**, Southern blot of EcoRI-digested DNA from FVII^{+/+}, FVII^{+/–} and FVII^{–/–} littermates probed with probe B, generating a 10.5 kb wild-type and a 6.1 kb disrupted FVII allele. **c**, **d**, Northern blot of FVII mRNA isolated from the liver from FVII^{+/+}, FVII^{+/–} and FVII^{–/–} neonates (**c**) or from the E9.5 embryo (lane 1), the E9.5 yolk sac (lane 2) or P2 neonatal (lane 3) or adult liver (lane 4) (**d**).

Table 1 Coagulant activities in FVII+/+, FVII+/- and FVII-/- mice

Coagulation factor	Age	FVII+/+	FVII+/-	FVII-/-
Factor II	E18.5	42 ± 4 (9)	39 ± 3 (21)	46 ± 4 (8)
Factor V	E18.5	45 ± 6 (9)	41 ± 4 (21)	60 ± 6 (8)
Factor VII	E11.5	0.2 ± 0.1 (11)	0.1 ± 0.1 (3)	≤0.05 (2)†
	E14.5	1.7 ± 0.3 (8)	0.9 ± 0.1* (11)	≤0.05 (9)†
	E18.5	39 ± 8 (3)	13 ± 2* (17)	≤0.05 (12)†
	neonates	23 ± 4 (12)	10 ± 2* (12)	≤0.05 (3)†
Factor VIII	E18.5	74 ± 15 (9)	62 ± 7 (21)	64 ± 8 (8)
Factor IX	E18.5	37 ± 4 (9)	28 ± 2 (21)	19 ± 2* (8)
Factor X	E18.5	30 ± 3 (9)	27 ± 2 (21)	34 ± 3 (8)

Data represent mean ± s.e.m. of *n* individual measurements (indicated between brackets) expressed as a per cent of adult wild-type levels.

**P* < 0.05 versus wild-type by analysis of variance.

† These values represent background chromogenic substrate hydrolysis activity by non-FVII-related mechanisms in the FVII-/- plasma.

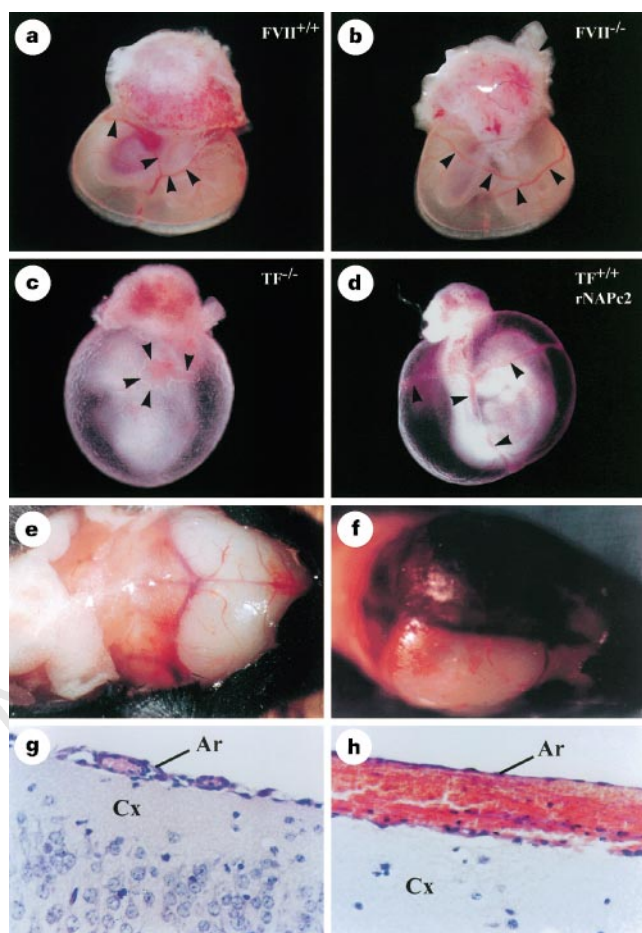


Figure 2 Normal vascular development and fatal postnatal bleeding in FVII-/- embryos. **a-d**, Macroscopic view of an E10.5 FVII+/+ (**a**), FVII-/- (**b**), TF-/- (reproduced from ref. 5) (**c**) and rNAPc2-treated TF+/+ (**d**) embryo, attached to their yolk sac, revealing large blood-filled vitello-embryonic vessels (arrowheads) and a dense capillary plexus in the FVII+/+ (**a**), the FVII-/- (**b**) and rNAPc2-treated TF+/+ (**d**) yolk sacs. In contrast, microaneurysmatic blood lakes (arrowheads) and an irregular vascular plexus with abnormal vitelline vessels are present in the TF-/- yolk sac (**c**). **a, b**, display freshly dissected and **c, d**, cultured embryos. **e, f**, Normal brain in a FVII+/+ mouse (**e**) and subdural haematoma in a FVII-/- animal (**f**). **g, h**, Sections from the same FVII+/+ (**g**) and FVII-/- (**h**) animals as in **e, f**, revealing blood in intact vessels between the arachnoid layer (Ar) and white brain matter (Cx) in the FVII+/+ animal (**g**) but subdural accumulation of blood in the FVII-/- animal (**h**).

rFVIIa was undetectable within the embryo even at supraphysiological maternal rFVIIa levels (for example, 10-fold higher than the endogenous ~500 ng ml⁻¹ FVII levels³) (Table 2). Beyond E10.5, rFVIIa was undetectable in the embryonic plasma at physiological maternal rFVIIa levels (~500 ng ml⁻¹) and only present at <0.1% of maternal rFVIIa levels at supraphysiological plasma levels (~5 µg ml⁻¹) (Table 2). These results indicated that transfer, if any, is very low. Because maternal-fetal transfer initially occurs through the yolk sac and after chorio-allantoic fusion (E9.0-9.5) primarily through the placenta⁹⁻¹¹, these sites were examined for the presence of FVII. At E9.5 or E10.5, FVII-ir was only detected in the visceral endoderm cells in the yolk sac from a FVII+/+ and from a FVII-/- littermate, primarily located at the villous brush border (maternal side) (Fig. 3b, c). In the E9.5 chorio-allantoic placenta (examined after injection of 2,500 µg kg⁻¹ rFVIIa for more sensitive detection but with similar topography of FVII-ir in the untreated female), FVII-ir was restricted to the endothelium of the maternal blood vessels and to the syncytiotrophoblast layer at the maternal-fetal interface (Fig. 3e, f). In contrast, the embryonic blood vessels were consistently FVII-negative (Fig. 3g). Because FVII gene inactivation precluded FVII synthesis, FVII-ir in FVII-/- visceral endoderm and trophoblasts probably resulted from adsorption or uptake of maternal FVII. Taken together, these results indicate that maternal-fetal FVII transfer can only be detected beyond E9.5 and at supraphysiological maternal FVII plasma levels. That FVII is detected on and within the visceral endoderm cells and syncytiotrophoblasts (but not within the rest of the embryo or yolk sac) may be attributable to their well known function to endocytose serum proteins, digest them into amino acids and transport these through the vitello-embryonic vessels to the embryo for nutrition⁹⁻¹¹. Lack of maternal-fetal transport of FVII¹² or of other coagulation factors¹³ has been previously suggested and contrasts to the efficient but highly specific transport of maternal immunoglobulin G¹⁴.

FVII binding to TF activates coagulation and induces fibrin formation¹⁻⁴, but TF may also be involved in other morphogenetic processes^{1,4,15}. Previous TF gene-inactivation studies proposed that bleeding from fragile TF-/- vessels following spontaneous embryonic vascular trauma was due to either defective fibrin formation and/or defects in periendothelial cell function⁵⁻⁸. Apparently, the immature E9.5 blood vessels are exposed to frequent vascular injury (related to increased haemodynamic stress), because multiple vessels spontaneously ruptured in each TF-/- yolk sac and bleeding occurred in 80 to 100% of TF-/- embryos. If fibrin formation was essential for haemostasis, it should be detectable around wild-type embryonic vessels. However, immunostaining (Fig. 3h) and ultrastructural analysis (≥20 optical fields of ×3,000 magnification per yolk sac in 5 wild-type E9.5 yolk sacs analysed; not shown) of wild-type E9.5 and E10.5 yolk sacs did not reveal fibrin in or around these vessels. Furthermore, if coagulation and fibrin formation were required for haemostasis at E9.5, FVII-/- embryos might be expected to bleed because their plasma FVII levels are undetectable (≤0.05% of adult levels). TF-FVII-initiated coagulation also did not appear to be essential for vascular integrity in cultured embryos. Indeed, culturing wild-type embryos from E8.5 to E10.5 (the critical period of vascular fragility in TF-/- embryos) in the presence of the recombinant active site FVIIa-inhibitor NAPc2¹⁶ (rNAPc2 which inhibits TF-FVIIa in a factor Xa-dependent manner; supplemented to the culture medium and daily injected intracardially) did not induce the typical TF-/- associated formation of microaneurysms, blood lakes and bleeding⁵ (Figs 2d, 3i, j), even when present at doses that were 100-fold (370 ± 160 nM; *n* = 6 embryos) to 1,000-fold (1,200 ± 450 nM; *n* = 7 embryos) higher than the ~1 nM required to inhibit TF-FVIIa activation¹⁶ or to induce bleeding in neonatal mice (Supplementary information Fig. S3). Notably, vascular fragility and bleeding were also not observed after rNAPc2 treatment of C57B16/J × 129/SvJ embryos (1,200 ± 250 nM; *n* = 4), indicating that the genetic background (and the theoretical influence of

Table 2 Maternal–fetal transfer of FVII

Age	Dose rFVIIa* (time sampling)	rFVIIa concentration			
		Plasma mother (ng ml ⁻¹)	Plasma embryo (ng ml ⁻¹)	Placenta (ng mg ⁻¹)	Embryo (ng ml ⁻¹)
E9.5	2,500 (1 h)	3,900 ± 550 (2)	Not done†	40 ± 3 (8)	N.D. (5)
E10.5	2,500 (1 h)	4,990 (1)	Not done†	Not done†	0.09 ± 0.03 (5)
E11.5	2,500 (1 h)	3,500 ± 630 (2)	2 ± 1 (9)	31 ± 3 (9)	0.04 ± 0.01 (9)
E14.5	500 (4 h)	70 (1)	ND (9)	0.1 ± 0.1 (9)	ND (9)
	2,500 (4 h)	1,200 (1)	ND (4)	4 ± 1 (4)	ND (4)
	2,500 (1 h)	4,400 ± 100 (2)	2 ± 1 (10)	6 ± 1 (10)	0.01 ± 0.01 (10)

The numbers represent measurements in the indicated number of embryos or mothers, expressed as ng ml⁻¹ plasma or as ng mg⁻¹ protein.

* Lower maternal rFVIIa plasma levels were obtained at 4 then at 1 h after injection.

† Not done, sampling of blood was only possible from embryos older than E10.5.

ND, not detectable. the detection limit of the assay was ≤50 pg ml⁻¹.

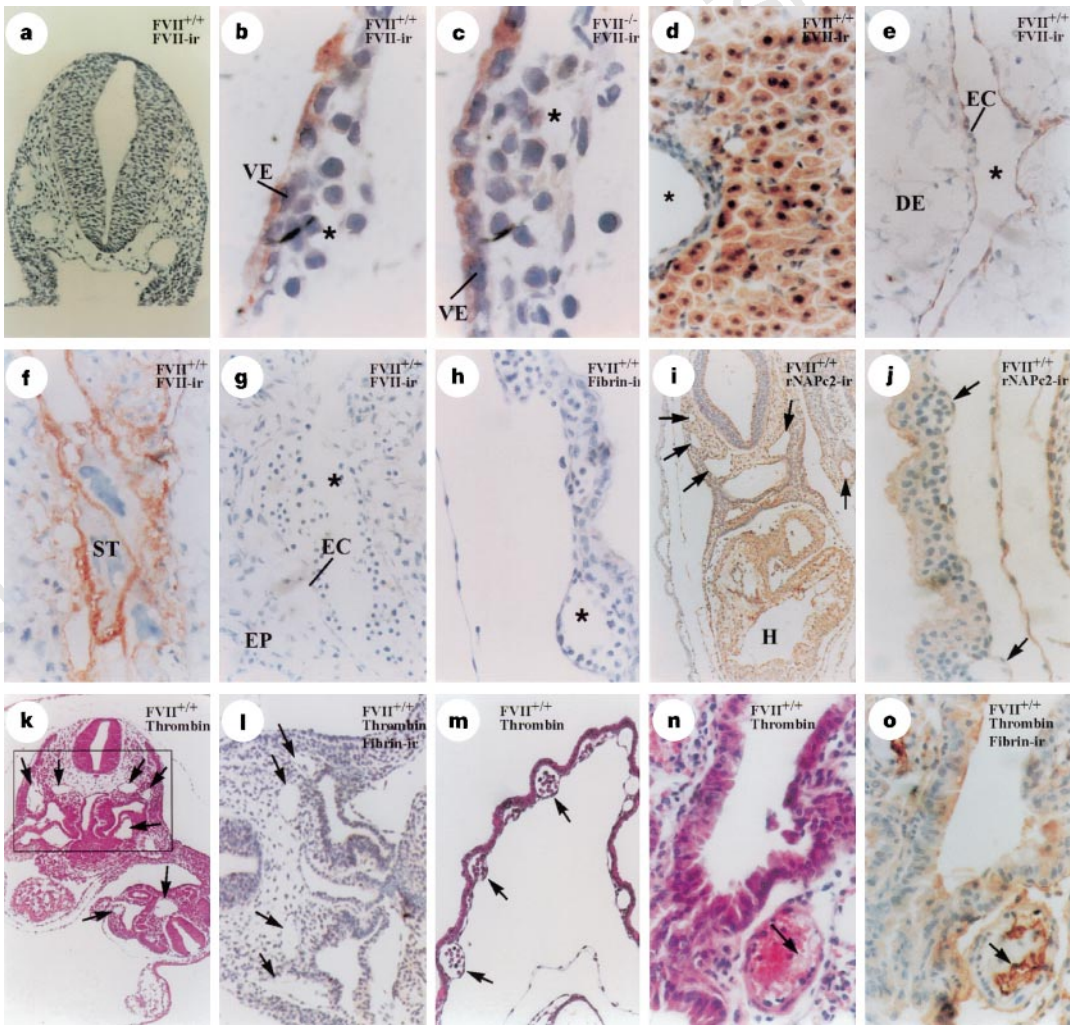


Figure 3 FVII and fibrin formation in embryos. **a–g**, FVII-immunoreactivity (ir) was absent in the E9.5 FVII^{+/+} embryo (**a**) but present in the apical region (maternal side) of the visceral endoderm (VE) cells in a E9.5 FVII^{+/+} (**b**) and FVII^{-/-} (**c**) yolk sac, and in the FVII^{+/+} adult liver (**d**). In the E9.5 placenta, FVII-ir was detected in maternal endothelial cells (EC) amidst maternal decidua (DE) (**e**), and on syncytiotrophoblasts (ST) (**f**), but not in embryonic blood vessels amidst embryonic placenta (EP) (**g**). Asterisks in **b–g**, **h**, denote the vascular lumen. **h**, Fibrinogen/fibrin-ir was not detected in or around E9.5 FVII^{+/+} yolk sac blood

vessels. **i, j**, NAPc2-ir was abundant throughout the embryo (**i**) and yolk sac (**j**) in a cultured E10.5 FVII^{+/+} embryo, treated for 2 days with rNAPc2. Note the normal vascular development (arrows indicate blood vessels; H, heart). **k–o**, Thrombin failed to induce fibrin thrombi in E9.5 wild-type embryonic (**k, l**) or yolk sac vessels (arrows) (**m**) but caused thrombosis in adult lung (arrows in **n, o**). **k, m, n**, Haematoxylin-eosin; **l, o**, fibrin(ogen) immunostaining. **l** displays the boxed region on an adjacent section of that shown in **k**. Sections in **n, o** are adjacent.

closely linked genes) was not critical. A possible explanation for these observations is that haemostasis in E9.5 embryos is minimally dependent on fibrin formation. This is also suggested by the surprising observation that fibrin clots were not detected in any of the embryonic or vitelline vessels (Fig. 3k–m) after intracardial injection of thrombin in E9.5 embryos, even at doses that were 100-fold ($n = 5$ embryos) to 1,000-fold ($n = 15$) higher than the $3,000 \text{ U kg}^{-1}$ required to induce lethal thrombosis consistently in adult mice¹⁷ (Fig. 3n, o). Platelet-dependent haemostasis may also not be essential because mature platelets have not been identified at E9.5 (S. Orkin, personal communication and ref. 18) and absence of platelets did not cause prenatal bleeding¹⁹.

In conclusion, FVII appears to be essential for perinatal haemostasis. Unexpectedly, embryonic FVII is not, whereas its cellular receptor TF is critical during development. Although it is possible that rNAPc2 was ineffective because of insufficient factor Xa levels and that FVII on the maternal side of the endoderm cells interacts with TF, TF–FVII-initiated coagulation in the yolk sac, if any, did not induce detectable fibrin formation and was not critical for vascular integrity. Maternal–fetal transfer of FVII if any, was below our detection limit ($\leq 0.05\%$ of adult levels or 5 pM). Therefore, it is highly unlikely that such low embryonic plasma FVII levels (of which $>99\%$ is probably inactive^{3,20}) would be adequate for fibrin-mediated haemostasis throughout the entire embryonic development. In fact, our data suggest that early embryonic haemostasis may be less dependent on TF–FVII-initiated fibrin formation than anticipated (see also Supplementary information Table S2), and raise the question whether developmental TF may be involved in alternative functions such as signalling^{4,15}, adhesion or chemotaxis²¹. □

Methods

Construction of targeting vector and gene targeting. The FVII targeting vector (pND.FVII; Fig. 1a) contained a FVII 5' flanking region consisting of a 4.0 kb *NotI*–*XhoI* fragment upstream of exon 2. The *XhoI* site was introduced by polymerase chain reaction (PCR) mutagenesis in exon 2, immediately to the 5' side of the sequence encoding alanine-1, the N terminus of the mature protein²². The FVII 3' flanking region consisted of a 5.7 kb *EcoRI*–*XbaI* fragment extending from a *NsiI* site in the 3'-untranslated region to a downstream *XbaI* site. A neomycin resistance (*neo^R*) and cytosine deaminase cassette (*cda*) were used for positive/negative selection. Targeted embryonic stem cells (RW4 cells from Genome Systems; derived from 129/SvJ strain²³) were used for aggregation with C57B16/J morula-stage embryos, and two FVII chimaeric mice, test-bred with C57B16/J mice, transmitted the inactivated FVII allele through the germ line. Numerous F1 FVII+/- founders were intercrossed through brother–sister mating to produce F2 littermates for phenotypic analysis, and breeding partners were regularly intermixed to minimize generation of partial congenic lines. Large groups of F2 offspring (>700 mice) were analysed to compensate for possible phenotypic differences resulting from F2 genetic background. To a limited extent, F2 FVII+/- offspring were intercrossed to generate F3 FVII-/- offspring. In those cases, several different F2 breeding pairs were intercrossed by brother–sister mating with frequent exchange of F2 partners between these breeding pairs. Although not included in the present study, the phenotype of the F3 FVII-/- offspring did not differ from that of the F2 FVII-/- offspring. F2 offspring from two independent FVII-targeted embryonic stem cell clones provided similar phenotypes.

Detection of FVII and other coagulation factors. FVII activity was measured using plasma prepared from anticoagulated blood by the Coatest FVII test (Chromogenix, Mölndal, Sweden). The coagulant activity of the other factors was determined as a clotting time after mixing the murine plasma with human plasma, deficient in the specific factor, and addition of thromboplastin (for FII, FV, FVII, FX) or kaolin (FIX). All procoagulant activities were expressed as a per cent of the procoagulant activity in a plasma pool of adult wild-type mice. Preparation of mRNA and northern blot analysis were as described²⁴ using a labelled murine FVII complementary DNA probe. For studying maternal–fetal transfer, 500 to $2,500 \mu\text{g kg}^{-1}$ recombinant human FVIIa (rFVIIa, Novo Nordisk, Gentofte, Denmark) was intravenously injected into timed mated

pregnant females. After 1 to 4 h, maternal and embryonic plasma (intracardiac puncture) and tissues were collected for FVII antigen determination using a human FVII-specific ELISA²⁵. FVII immunostaining was performed on 1% paraformaldehyde-fixed cryosections with a crossreacting rabbit anti-human FVII polyclonal antiserum²⁶. Specificity of the staining was demonstrated using preimmune serum, omission of the primary or secondary antibody layers, preadsorption of the primary antiserum with rFVIIa or staining of FVII-/- liver sections, which each abolished specific staining (not shown). Immunostaining for smooth muscle α -actin, von Willebrand Factor and fibrin(ogen) was as described²⁷.

Embryo culture and histology. Culture of embryos from E8.5 to E10.5 (NMRI strain) or to E9.75 (C57B16/J $\times$ 129/SvPas strain, obtained by timed mating of TF+/+ mice⁵), was as described²⁸. Injections in the bulbo-ventricular canal of the heart of E8.5 or E9.5 embryos used injection pipettes (diameter $\sim 10 \mu\text{m}$; drawn from 1-mm glass capillaries using a Narishige PN-3 microforge) under an inverted microscope (Narishige/Nikon Diaphot 200), equipped with micro-manipulators (Narishige MM-188 and Nikon MO-188) and a Miki injector (S-01). Roughly 0.5 to $1.0 \mu\text{l}$ of saline, rNAPc2 (10 or $100 \mu\text{M}$) or thrombin ($1,250$ or $9,000 \text{ U ml}^{-1}$; $2,500 \text{ U mg}^{-1}$) solutions (both diluted in saline) were injected under continuous positive flow to avoid contamination with the surrounding fluids. For injections of thrombin, E9.5 embryos were dissected and immediately injected while in prewarmed saline. Adult mice received thrombin ($3,000 \text{ U kg}^{-1}$) via intravenous injection in the jugular veins. Control injections of vital dyes revealed homogenous distribution throughout the embryo. All other methods are described elsewhere^{24,29}.

Received 18 July; accepted 28 August 1997.

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Supplementary Information is available on Nature's World-Wide Web site (<http://www.nature.com>) or as paper copy from Mary Sheehan at the London editorial office of Nature.

Acknowledgements. We thank U. Hedner and M. Ezban (Novo Nordisk, Gentofte, Denmark) for the gift of rFVIIa, P. Bergum (Corvas, San Diego, CA) for rNAPc2 measurements, J. W. Fenton (Albany, NY) for thrombin, T. Edgington and E. Conway for discussions and M. Dewerchin, V. Attenburrow, A. Bouché, I. Cornelissen, S. Danloy, F. Decock, M. Deprez, M. DeWit, E. Gils, L. Kieckens, A. Manderveld, A. Vandenhoek, A. Van den Boomen and S. Wyns (CTG); J. Picard and N. Pacico (LDG) and H. Fehrenbach (IP) for help. These studies were supported by a grant from the National Institutes of Health and the Kleiderer/Pezold Family endowed professorship (to F.J.C.). J.C.Y.C. is a recipient of the Raymond Tower Endowed Graduate Fellowship.

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Binary specification of the embryonic lineage in *Caenorhabditis elegans*

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In *Caenorhabditis elegans*, the early embryo contains five somatic founder cells (known as AB, MS, E, C and D) which give rise to very different lineages. Two simply produce twenty intestinal (E) or muscle (D) cells each, whereas the remainder produce a total of 518 cells which collectively contribute in a complex pattern to a variety of tissues¹. A central problem in embryonic development is to understand how the developmental potential of blastomeres is restricted to permit the terminal expression of such complex differentiation patterns. Here we identify a gene, *lit-1*, that appears to play a central role in controlling the asymmetry of cell division during embryogenesis in *C. elegans*. Mutants in *lit-1* suggest that its product controls up to six consecutive binary switches which cause one of the two equivalent cells produced at each cleavage to assume a posterior fate. Most blastomere identities in *C. elegans* may therefore stem from a process of stepwise binary diversification.

Because early blastomeres establish regions in the *C. elegans* embryo, it was proposed that the complexity of the lineage produced by the somatic founder cells AB, MS and C evolved to satisfy the local requirements for tissue types within these regions^{1,2}. Although much progress has been made in identifying genes involved in the specification of the embryonic lineage³, the question of how such a complex lineage can be specified remains open.

Mutations in *lit-1* which cause maternal-effect embryonic lethality (loss of intestine; *t1512* and *t1534*) were identified in a screen for parental effect lethal mutants. The gene is also required zygotically in the embryo and during larval development (see Methods). The gene appeared to be interesting because embryos from mothers homozygous for the temperature-sensitive strict maternal effect allele *t1512* completely lack intestinal cells but have an excess of pharyngeal cells (Table 1a, e and Fig. 1a–f), which suggests that the E fate, a posterior fate, may be transformed to an anterior MS fate. Laser ablation experiments confirm such a transformation: E does not produce any intestinal cells but instead gives rise to 15 pharyngeal and 4 body-wall muscle cells (Table 1b) a combination of cell types only occurring in MS. Further analysis showed that not only E but all somatic founder cells except for D are affected in these embryos. The AB blastomere produces only 2 instead of 7 specific pharyngeal cells and 6 instead of 20 hypodermal seam cells (Table 1a, c). MS produces only 3 instead of 28 body-wall muscle cells, but the normal number of 14 pharyngeal cells is raised to 24 (Table 1c, d; Fig. 1g, h). Body-wall muscle cells are also significantly reduced from 32 to ~9 in the C blastomere but are normal in D (Table 1d). Thus the founder cells AB, MS and C are still able to express the characteristic tissues but in altered numbers. The alterations cannot be explained by assuming that *lit-1* has a specific function in the determination of tissues like hypodermis or muscle.

Using a four-dimensional microscope², we learned that *lit-1* (*t1512*) causes very early alterations in the E lineage. The blastomeres Ea and Ep do not gastrulate initially. Both blastomeres divide anterior–posteriorly instead of left–right. The cell cycle timing of their descendants is identical to that of MS (Fig. 2a). Also, the terminal differentiation patterns of the E lineage show specific features of the MS lineage, including the exhibition of the prominent first cell death in the embryo (Fig. 2b). However, the lineage of the transformed E blastomere is not completely identical to a MS lineage, but is composed in a ‘copy-and-paste’ manner of MS sublineages. Anterior sublineages are not only expressed in their normal positions but also in more posterior positions. For example, the Eapaa lineage does not look like that of its lineage homologue MSapaa, but like that of MSaaaa, a more anterior lineage (Fig. 2b, d). These observations raised the possibility that the complexity of the *lit-1* phenotype may be explained by multiple posterior-to-anterior transformations, as observed in the EMS lineage. E is first transformed into MS, and further posterior-to-anterior transformations occur in this second MS lineage in the embryo.

To determine whether such anterior–posterior transformations may also explain the lack of body-wall muscle observed in C, we also analysed this lineage. The four granddaughters of C produce either mainly hypodermal cells (Caa and Cpa) or body-wall muscle cells (Cap and Cpp). One cleavage later, the lineages of the great-granddaughters Caaa and Caap can again be distinguished because Caap produces not only hypodermis, but also neurons and a cell death

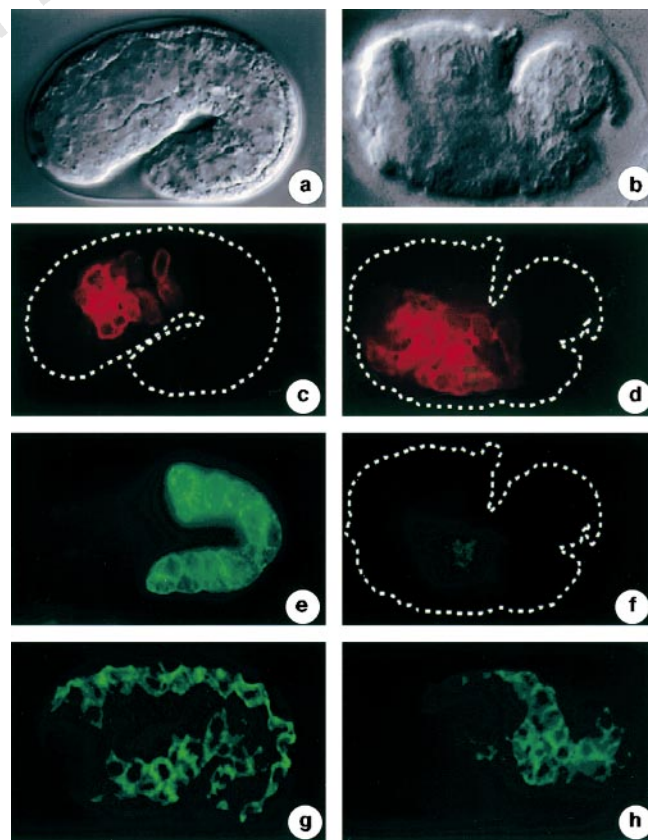


Figure 1 Tissue differentiation in wild-type (a, c, e, g) at the 1.5-fold stage of embryogenesis¹ and in two *lit-1* (*t1512*) embryos (one embryo, b, d, f; the second, h). a and b, Normarski images. b, *lit-1* (*t1512*) embryos show a partial morphogenesis and often form a little tail. c and d, Embryos stained with monoclonal antibody (mAb) 3NB12 (ref. 22) recognizing pharyngeal muscle cells. d, We counted 36 pharyngeal cells instead of the normal 21 in this embryo. e and f, Embryos stained for intestine with mAb ICB4 (ref. 23). f, The same embryo as in d does not show any intestinal differentiation. g and h, Embryos stained with mAb NE8 4C6.3 (antibody collection MRC LMB, Cambridge) recognizing 81 body-wall muscles in wild type. h, We counted 36 body-wall muscle cells in this mutant embryo.

(Fig. 2c). The lineage alterations in different *lit-1* embryos can generally be ordered in a phenotypic series. In weak phenotypes, Caa is normal but Cap executes a Caa pattern (Fig. 2c). In the next stronger phenotype, the same transformation is seen, but in addition a posterior-to-anterior transformation in the Caa lineage occurs after the next cleavage. The Caap lineage is replaced by the Caaa lineage. The strongest phenotype corresponds to only-anterior fate, the expression of hypodermis in the whole lineage. Posterior lineages in all phenotypes are replaced by anterior ones. Thus it appears that with decreasing activity of *lit-1*, consecutive posterior-to-anterior transformations on different levels of the lineage occur. Owing to the specifics of the C lineage, it is not possible to determine whether any posterior-to-anterior transformations occur beyond the level of Cxxx (sixth cleavage round). However, the MS lineage, in which similar posterior-to-anterior transformations are observed, provides more detail in the lineage pattern, which brings the score of posterior-to-anterior transformations up to the level of the MSaaaa blastomere (eighth cleavage round; Fig. 2d).

As already discussed, the AB lineage is also affected in *lit-1*

mutants. All 8 AB blastomeres present at the 12-cell stage are affected, but their normal identity appears to be generally retained (Fig. 2e, legend). We investigated the ABara (data not shown) and ABpla lineages in detail as the differentiation patterns of those lineages are especially suited to score posterior-to-anterior transformation. As in the MS lineage, such transformations were observed up to the eighth cleavage of the embryo (Fig. 2e).

Our lineage analysis suggests that *lit-1* is required earliest in the EMS lineage (4-cell stage) and then for the specification of the complex AB, MS and C lineages up to the 256-cell stage, but not in the E and D lineages. The temperature-sensitive phase of *t1512* indicates a continuous requirement for *lit-1* activity up to this stage (Fig. 3). An analysis of the differentiation of the EMS lineage in embryos in which *lit-1* activity was restored at different developmental stages by temperature downshifts demonstrates that the gene is repeatedly required for stepwise anterior-posterior decisions. A downshift at the 2-cell stage restores the formation of intestine, then the pharynx increases at the expense of intestine. A downshift immediately after the E-to-MS transformation still permits the expression of the posterior fate of body-wall muscle.

Table 1 Immunochemical analysis of tissues in mutant *lit-1* embryos

(a) Tissue differentiation in <i>lit-1</i> mutant embryos				
Allele	Pharynx muscle		Intestine	Body-wall muscle
Wild type	21		20	81
<i>t1534</i>	23 ± 4 (37) 18–32		20 ± 1 (34) 18–21	45 ± 6 (31) 28–54
<i>t1512</i>	37 ± 6 (106) 25–52		0 ± 3 (106) 0–10	35 ± 10 (51) 20–40
<i>t1512/tDf6</i>	42 ± 9 (23) 29–61		0 ± 1 (20) 0–1	23 ± 10 (23) 16–36
Seam cells	Wild type: 20		<i>t1512</i>	6 ± 3 (54) 1–12
(b) E produces pharyngeal and body-wall muscle cells				
Isolated	Ablated	Exp	Pharynx muscle	Body-wall muscle
E	ABa/p, P2, MS	0	15 ± 2 (10) 12–20	4 ± 2 (10) 0–7
(c) Blastomeres contributing to pharynx muscle				
Isolated	Ablated	Exp	Wild type	<i>t1512</i>
ABp, P2	ABa, EMS	0	0 (6)	0 (15)
ABp, P2	ABa, MS, E	0	ND	0 (10)
EMS	ABa	14	13 ± 1 (8) 12–14	34 ± 4 (14) 27–41
EMS	ABa, ABp, P2	14	ND	38 ± 5 (9) 33–47
ABa, E	MS	7	7 ± 0 (8) 6–7	17 ± 4 (6) 10–20
ABa	MS, E	7	6 ± 1 (5) 5–7	2 ± 2 (14) 0–5
E	ABa, MS	0	0 (9)	14 ± 3 (11) 10–17
MS	ABa, E	14	13 ± 1 (5) 12–14	24 ± 2 (14) 20–26
MSa	ABa, E, MSp	8	8 ± 0 (4)	14 ± 2 (6) 12–15
MSp	ABa, E, MSa	6	5 ± 1 (4) 4–6	11 ± 2 (6) 8–12
MSaa	ABa, E, MSp, MSap*	8	ND	9 ± 1 (6) 8–9
MSap	ABa, E, MSp, MSaa*	0	ND	6 ± 1 (10) 5–7
MSpa	ABa, E, MSa, MSpp*	6	ND	6 ± 1 (5) 5–7
MSpp	ABa, E, MSa, MSpa*	0	ND	4 ± 1 (8) 2–6
ABp, P2	ABa, E, MSa, MSpx*	0	ND	0 (8)
(d) Blastomeres contributing to body-wall muscle				
Isolated	Ablated	Exp	Wild type	<i>t1512</i>
ABa/p	EMS, P2	1	0 ± 1 (3)	0 (7)
EMS	P2	29	28 ± 2 (10) 26–30	8 ± 3 (20) 3–13
E	P2, MS	0	ND	5 ± 2 (10) 2–8
EMS, C	P3	61	57 (13) 44–62	17 ± 5 (10) 8–23
(e) Comparison of <i>t1512</i> and <i>t1534</i> referring to muscle				
Isolated	Ablated	Exp	<i>t1512</i>	<i>t1534</i>
EMS	P2	28	8 ± 3 (20) 3–13	14 ± 4 (7) 9–20
EMS, C	P3	61	17 ± 5 (10) 8–23	33 ± 6 (7) 21–40
(f) Tissue differentiation in <i>lit-1</i>; <i>pop-1</i> mutant embryos				
Allele			Pharynx muscle	Intestine
Wild type			21	20
<i>unc-32 lit-1</i> ; <i>dpy-5 pop-1</i> ++ 25°C			37 ± 4 (10) 31–43	0 ± 1 (10) 0–2
<i>unc-32 lit-1</i> ; <i>dpy-5 pop-1</i> 15°C			8 ± 3 (10) 6–14	39 ± 4 (10) 31–46
<i>unc-32 lit-1</i> ; <i>dpy-5 pop-1</i> 25°C			8 ± 3 (18) 4–15	36 ± 5 (18) 28–50

a, Tissue differentiation in *lit-1* embryos (antibodies, see Fig. 1; seam cells are recognized by NE2-1B14 (ref. 21)). **b–e**, Laser ablation experiments of *lit-1* embryos (*t1512*). The blastomeres (isolated) were analysed after ablation of all other blastomeres (ablated) normally contributing to a tissue. Exp, the expected number of cells in a wild-type embryo after the same manipulation¹². Mean of counts ± standard deviation of stained cells is shown. The number of embryos (in brackets) is followed by the lowest and highest count of a series. **b**, The E blastomeres of *t1512* embryos that were isolated by ablating all other blastomeres produce pharynx muscle and body-wall muscle. **c**, In wild-type only the ABa (7 cells) and the MSxa blastomeres (14 cells) produce pharyngeal muscle cells, but in *lit-1* (*t1512*) embryos E and MSxp but no other cells produce them. EMS in *t1512* embryos produces more than twice the normal amount of pharynx muscles, irrespective of the blastomeres ablated. ABa produces less pharynx muscles in *lit-1* embryos. **d**, The transformed E blastomere as well as MS and C produce less body-wall muscle cells than expected. EMS produces only 8 of the normal 28 body-wall muscle cells. E alone produces ~5 of the 8 muscle cells so very few (3) can be derived from MS. We estimate from the difference between P2 and P3 ablated embryos that C contributes to about 9 of the 17 muscle cells found in *t1512* embryos. D-derived body-wall muscle seems to be unaffected, as revealed by examination of the partial lineage of two embryos of *t1534*, three of *t1512* and five of *t1512/tDf6*. **e**, In the weak allele, more muscle differentiates than in the strong allele. The E blastomere of the weaker allele *t1534* exhibits no MS like fate (**a**, confirmed by lineage analysis of two embryos). Nevertheless, the MS blastomere produces only 14 of the normal 28 body-wall muscle cells. MS and C together produce 33 muscle cells, therefore C produces only 19 cells. This agrees with lineage analysis showing that the posterior-to-anterior transformations that remove muscle are less frequent in the weaker allele. ND, not determined.

The later the function is restored after this, the more pharyngeal (anterior) fate is expressed at the expense of body-wall muscle (posterior fate) (Fig. 4).

It is known that the E blastomere depends on a cell-cell interaction polarizing EMS^{4,5} and that E develops like MS in its absence.

lit-1 generally affects the polarity of not only EMS, conferred by an induction, but also of lineages that develop autonomously^{6,7}. Therefore *lit-1* may function in the interpretation of the cell polarity, independently of whether the polarity arises autonomously or non-autonomously⁸. A recessive mutation in the gene *pop-1* causes a

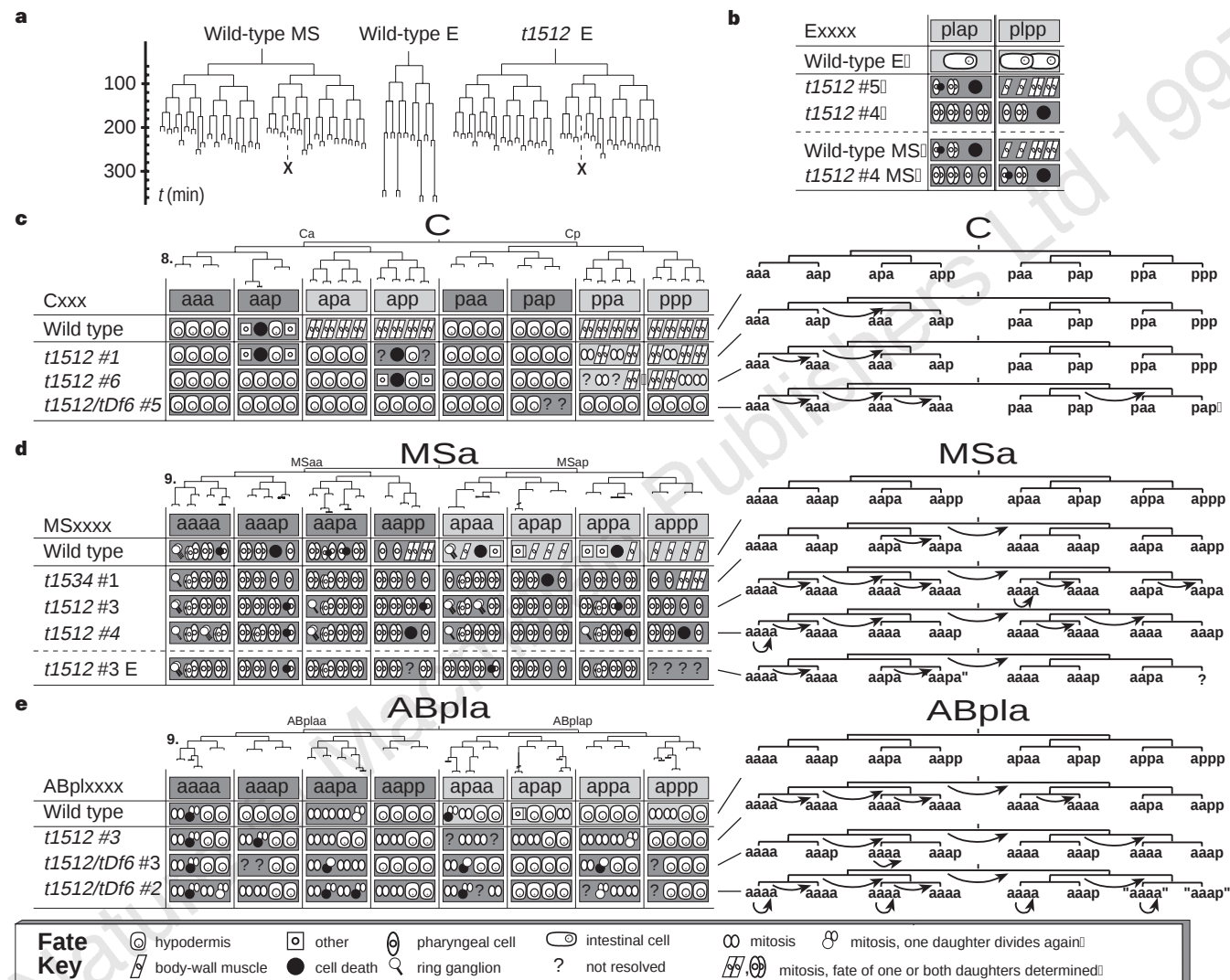


Figure 2 Lineage analysis of *lit-1* embryos with a four-dimensional microscope². **a**, Timing of cell cleavages of normal and transformed lineages derived from EMS. The wild-type lineages represent the mean of two, those of the mutant (*t1512*) the mean of three embryos. The timing of cleavages in wild-type and mutant lineages varies in the range of 1–8% (see also ref. 2). The E-lineage in mutant (*t1512*) embryos execute an MS-like lineage pattern. Owing to multiple successive posterior-to-anterior transformations, the Ea-derived lineage repeats four times a cleavage pattern similar to MSaaa. **b–e**, Analysis of terminal cell fates. Lineage data are shown in the fate tables. For **c–e**, our interpretations of the observed transformations are illustrated in schematic lineage diagrams beside the tables. The first row of each table shows the blastomere names (for example, Cxxx) from which the analysed lineage pattern is derived. Cell fates and mitosis patterns used to identify lineages are shown in the key. As morphogenesis is aberrant in the mutant embryos, it was sometimes only possible to score the fate of one of the daughters after a mitosis (44 of 110 cases). *lit-1* embryos are shown according to their phenotypic strength. We observe that the phenotype of the strong allele *t1512* is slightly stronger *in trans* to a deficiency. The immunochemical analysis (Table 1a) corroborates our conclusion that *t1512* does not define the null phenotype. **b**, In *lit-1* embryos, E-derived cells execute fates typical for the MS lineage. For example, the prominent cell-death MSpaapp is present at the corresponding position (Eplapp) in mutant *t1512* (4 of 5) and *t1512/tDf6* embryos (3 of 6). In addition, this cell death is found in another ectopic position, Eplppp of

t1512 (1 of 5) and *t1512/tDf6* embryos (2 of 6). Similar transformations are also seen in the MSp blastomere of mutant *t1512* embryos (3 of 5). **c**, In the C lineage of *lit-1* embryos, the body-wall muscle and the cell death in Caap are replaced by hypodermis (*t1534*, 2 of 3 and *t1512*, 5 of 5 only Ca; *t1512/tDf6*, 4 of 5 Ca and Cp). The changing position of the cell death indicates a replacement of Caxx by Caaa in three posterior-to-anterior transformations. **d**, In MS the posterior-to-anterior transformations extend, as indicated by the arrows, within the MSaaaa lineages to MSxxxxx (eighth cleavage). The MS lineages of three additional embryos were at least partially analysed and showed similar transformations. The last row of this panel shows, as an amendment to **b**, the complete lineage of the Ea blastomere of the embryo *t1512* #3. The whole lineage is transformed to execute anterior but not posterior MS-like fates. **e**, Posterior-to-anterior fate transformations in the ABpla lineage. Because of the posterior-to-anterior transformations in *lit-1*, it is difficult to assess the basic identities of the 8 AB blastomeres (12-cell stage). Lineage analysis and tissue expression suggest that seven of the eight AB descendants execute their basic identities. Only the ABarp lineage (3 embryos) appears aberrant in a way that cannot be explained by posterior-to-anterior transformations similar to ABpla. As this blastomere is most sensitive to a perturbation of the polarizing induction at the 2-cell stage establishing the ABxp identities²⁴, we cannot exclude the possibility that this induction also depends on *lit-1*.

transformation opposite to that by *lit-1*: E is not transformed into MS but MS into E. *pop-1* is thus required to promote anterior fate, whereas *lit-1* promotes posterior fate in the EMS lineage⁹. To determine the epistatic interaction between the two genes, we analysed embryos from doubly mutant mothers. These embryos have the *pop-1* phenotype (Table 1f), which suggests that *pop-1* functions downstream of *lit-1* and is required for the execution of anterior fate in *lit-1* mutants. This can be explained by assuming that *lit-1* normally suppresses *pop-1* activity in the posterior E cell. The zygote P0, which divides in an anterior–posterior direction, appears like the other germline precursors (P1–P3) to be unaffected in the *lit-1* mutants. These divisions are known to depend on the *par* genes¹⁰. This suggests that two independent pathways establish asymmetry either in the germline or in the soma.

In summary, our results indicate that *lit-1* repeatedly functions in the establishment of asymmetry of cell fates from the third to at least the eighth of the ~9–10 cell-division cleavages occurring in the *C. elegans* embryo. The phenotypes of the mutants described here show that posterior-to-anterior transformations can occur independently of each other in different lineages and at different stages of development. *lit-1* does not function at the level of tissue specification as do the myogenic genes, for example¹¹, but causes transformations at the level of blastomere identity¹². The eight great-granddaughters of AB, whose identity appears normal in the *lit-1* mutants described here, are also specified at the level of

blastomere identity by three binary switches conferred by induction¹². We propose that in *C. elegans*, most of the lineage is built by a stepwise binary diversification of blastomere identities. This general strategy for cell specification in early embryogenesis appears to be very different from that in *Drosophila*, where regional identities are established using a multitude of gradients^{13,14}. In later processes, however, binary specifications of cell fates depending on the genes *numb* and *prospero* occur also in certain tissues of the fruitfly and of the mouse¹⁵, or in *C. elegans*, depending on *lin-12* for example¹⁶. □

Methods

Genetics. The Bristol strain N2 was used as the standard wild-type strain. The following genes and alleles were used: *qC1* [*dpy-10(e1259) glp-1(q33)*], *unc-32(e189)*, *unc-71(ay7)*, *bli-5(e518)*, *him-3(e1147)*, *tDf5*, *tDf6*, *plg-1(e2001)*, *him-5(e1940)*, *pop-1(zu189)*, *dpy-5(e61)*. Some of these strains were obtained from the *C. elegans* Genetic Center, which is funded by the NIH National Center for Research Resources (NCRR). The *lit-1* alleles were obtained from a screen of 15,600 chromosomes for parental-effect embryonic lethal mutations on LGIII balanced by *qC1* (H.S. and R.S., unpublished results). Both alleles were outcrossed twice and maintained as heterozygotes (*unc-32 lit-1/qC1; him-3*). *t1512* and *t1534* failed to complement each other. Both alleles are uncovered by the deficiencies *tDf5*, *tDf6* and *ctDf2*. By three-factor cross between *unc-71* and *bli-5*, *t1512* was mapped to ~0.3 mU left of *bli-5* (data submitted). Embryos from heterozygous *t1512/t1534* or hemizygous (*lit-1/Df*) mothers and the *unc-71 lit-1(t1512)* strains from the three-factor cross all show the typical *lit-1* phenotype. *t1512* is a temperature-sensitive mutation. Homozygotes are viable at 15 °C. At 20 °C, heterozygous (*unc-32 lit-1(t1512)/qC1; him-3*) animals segregated 25% Uncs and 6% non-viable embryos ($n = 926$; n is number of progeny counted), all Uncs produce 100% dead eggs ($n > 500$). Control animals (*unc-32/qC1; him-3*) segregated 20% Uncs and 3% non-viable embryos ($n = 2,371$; brood size 181 ± 38 ; 5 hermaphrodites). At 25 °C, *t1512* heterozygotes segregated as 11% Uncs and 15% non-viable embryos ($n = 1,220$; brood size 146 ± 21 ; 8 hermaphrodites). These *unc-32 lit-1(t1512)* homozygotes grow up as sterile or, possibly owing to a neuronal defect, egg-laying-defective animals. *t1534* is a non-conditional mutation. At 20 °C *unc-32 lit-1(t1534)/qC1; him-3* animals segregated 21% Uncs and 13% non-viable embryos ($n = 495$). At 25 °C, 21% Uncs and 12% non-viable embryos were produced ($n = 682$; brood size 143 ± 35 ; 5 hermaphrodites). All Uncs tested at both temperatures produced only dead eggs ($n > 750$). The brood size of Unc L4 larvae shifted to 25 °C and homozygous for either allele linked to *unc-32* was the same (*t1512*, 160 ± 26 , $n = 5$; *t1534*, 154 ± 17 , $n = 5$) as of *unc-32* alone (146 ± 23 , $n = 5$). These data suggest that the null phenotype of *lit-1* could be zygotic embryonic lethal. *t1512* is strict maternal-effect lethal: that is, homozygous mothers crossed with *plg-1; him-5* males¹⁷ still produce only non-viable embryos. *t1534* is non-strict, because in the same cross *t1534* mothers produce embryos of which 40% hatch, but only 10% of these grow up to fertile adults. This provides further evidence for a zygotic function for *lit-1*. Embryos for phenotypic analysis were produced by growing worms at either 15 °C or 20 °C, followed by shifting homozygous L4 hermaphrodites to 25 °C where they were allowed to lay eggs.

Epistasis to *pop-1*. *pop-1(zu189) dpy-5; unc-32 lit-1(t1512)* mothers were always derived from single *pop-1 dpy-5/++*; *unc-32 lit-1* animals at 15 °C. Unc animals of the brood were shifted to 25 °C to ascertain the presence of *t1512*. The phenotype of embryos from DpyUnc animals raised at 15 °C was analysed to confirm the presence of *pop-1*. DpyUnc L4 larvae were shifted to 25 °C to determine the phenotype of the embryos from mothers homozygous for all mutations.

General methods. All methods have been described^{2,18–20}.

Received 22 July; accepted 21 October 1997.

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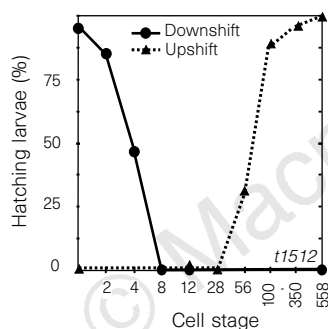


Figure 3 The temperature-sensitive period of *lit-1* (*t1512*). The activity is required from the 2-cell to approximately the 100-cell stage. Each point corresponds to at least 14 (on average 34) embryos.

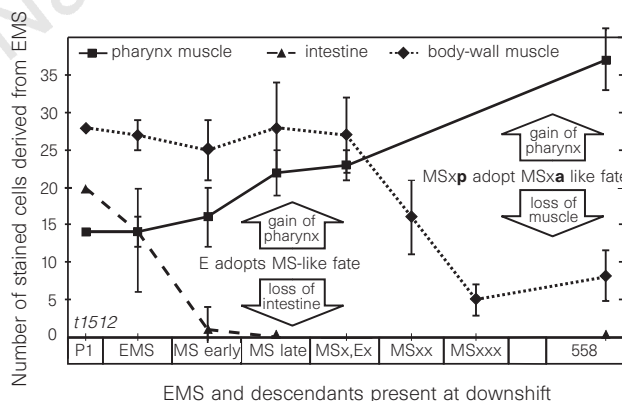


Figure 4 Requirement of *lit-1* at multiple stages during development. Mutant embryos (*t1512*) were laser-ablated at 25 °C (except the 2-cell embryos) and down shifted to 15 °C at given cell stages (except the 558-cell embryos). To score for EMS-derived pharynx and intestine, ABA was ablated; to score for EMS-derived muscle, P2 was ablated. At least five, on average 8, embryos were analysed for each stage and tissue (for control experiments, see Table 1c, d). The later the activity is restored (P1 to MSxxx) the more pharynx muscle (anterior fate) and the less intestine and body-wall muscle (posterior fates) are expressed.

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A non-retroviral RNA virus persists in DNA form

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Infection of adult mice with lymphocytic choriomeningitis virus (LCMV), a non-cytopathic segmented RNA virus, leads initially to generalized infection, followed by clearance and subsequent life-long immunity. Indirect evidence has suggested that viral antigens may persist in lymphoid tissues during the phase of immunological memory, but viral genomic RNA has not been detected in previous studies^{1,2}. During a search for persistent virus in the spleen, we identified LCMV-specific sequences present as DNA by polymerase chain reaction (PCR) in mice over 200 days after infection. *In vivo* and *in vitro* studies revealed that reverse transcription of viral RNA into complementary DNA occurred after acute infection of cells of its natural hosts, mouse and hamster, but not of other species and could be inhibited *in vitro* by azidothymidine (AZT), indicating that this was mediated by endogenous reverse transcriptase activity. These findings reveal a surprising and new pathway of interaction between exogenous RNA viruses and endogenous retroviral, and perhaps other host components, that results in the persistence of virally determined DNA. We speculate that the latter may function *in vivo* as a form of DNA vaccine.

Although earlier studies have suggested that LCMV-derived antigens may persist for long periods in the host^{3,4}, recent attempts to demonstrate infectious virus or viral RNA in lymphoid tissue after acute low-dose infection have failed^{1,2,5}. To analyse further whether viral material may be present in some form in spleens or lymph nodes after clearance of acute infection, mice were infected with LCMV (200 plaque-forming units; WE strain) and then tested

for the persistence of LCMV-specific sequences existing as either RNA or DNA. Such mice cleared virus from the blood within 2 weeks, and spleens from immune animals 60–200 days after infection were uniformly negative for culturable virus using standard assays⁶: even activation of lymphocytes by mitogens including lipopolysaccharide (LPS), phytohaemagglutinin (PHA) and staphylococcal enterotoxin A did not lead to release of infectious virus into the supernatant (data not shown). Consistent with previous reports, RNA extracted from splenocytes and reverse transcribed *in vitro* was also negative using a standard LCMV glycoprotein (GP)-specific PCR^{2,7}.

Surprisingly, however, splenocyte DNA which was treated with RNase and then subjected directly to PCR specific for LCMV GP or nucleoprotein (NP) (Fig. 1a) was positive in 11/18 and 10/15 immunized mice, respectively (C57BL/6; 200 PFU LCMV-WE intravenously (i.v.)). Figure 1b illustrates an experiment performed using a set of nested primer pairs directed at NP, in which control mice were negative, but 2/2 mice challenged with LCMV 70 days previously were positive. Using separate primer pairs and a hot start procedure, products were also amplified from a separate set of immune mice using a single round of PCR (Fig. 1c). The material was insensitive to further RNase A or H treatment before PCR amplification but sensitive to treatment with double-strand-specific DNase (Fig. 1d) confirming its identity as DNA. Nested PCR for a spliced form of perforin⁸ was negative in the same splenocyte DNA, although positive in splenocyte RNA reverse-transcribed *in vitro* (Fig. 1e), which indicates that *in vivo* such cDNA forms of expressed genes are not universally found, and further that the LCMV DNA forms found are not an artefact of reverse transcription by Taq polymerase *in vitro*. A similar result was obtained with a single-step PCR for beta-actin cDNA (data not shown)⁸.

The GP-specific PCR was able to detect three copies of template per µg cellular DNA and products could be identified in DNA extracted from a minimum of 3×10^4 to 3×10^5 spleen cells from immune mice, yielding an estimate of 1 copy per 10^4 to 10^5 splenocytes. A GP-specific band was also found in 2/5 BALB/c and 1/4 wild (non-laboratory-bred, farm-caught) mice infected as above (200 PFU LCMV-WE i.v.), but not in uninfected spleens.

To further investigate the generation of this virally determined DNA, the infection of susceptible cells lines was studied *in vitro*. Specific viral DNA was first seen 8–10 h after infection of MC57 cells (a transformed murine fibroblast line), and appeared to accumulate in DNA extracted after this time (Fig. 2a). Pretreatment of MC57 cells with azidothymidine (AZT) in a manner analogous to its use with HIV-1 *in vitro*⁹ led to a dose-dependent inhibition of DNA formation (Fig. 2b). The presence of AZT did not affect the PCR amplification of a genomic perforin sequence from the same cellular DNA (Fig. 2c) or the level of virus production in the supernatant (6.1 – $6.4 \log$ PFU ml⁻¹). Treatment of infected MC57 cells with actinomycin D, a drug that interferes both with second-strand synthesis during reverse transcription and with arenavirus replication¹⁰, also led to inhibition of PCR product formation in doses between 1 and 5 µg ml⁻¹ (data not shown).

As a further control for specificity, PCR products from DNA extracted from MC57 cells infected *in vitro* with LCMV strains Armstrong or WE were amplified and directly sequenced. These were compared with sequences from products derived from infected B-cell hybridomas derived *ex vivo*¹¹, and from immune mouse spleen. Figure 2d confirms that the products were LCMV strain-specific.

Although a range of mammalian lines (including human, monkey, dog and cow) were susceptible to infection with LCMV as judged by comparable production of culturable virus in the supernatant and by fluorescence-activated cell sorting (FACS) staining for surface-expressed glycoprotein, only lines derived from mouse (MC57 fibroblast, L929 cells and IC21 monocyte-derived) and hamster (baby hamster kidney cells) were found to be positive for LCMV-specific DNA by PCR (Table 1). This was found irre-

spective of whether the infecting LCMV was derived from stocks grown on murine, monkey or canine cells. Because all of the above lines contained viral RNA, this further demonstrates that the PCR product cannot be due to reverse transcription and amplification by Taq polymerase, acting on any residual RNA left after RNase treatment. Correspondingly, only mice and hamsters are thought to be natural hosts for LCMV and these, but not other species, may become permanent virus carriers¹².

In vivo, in addition to mice, in 2/2 hamsters infected with LCMV (2×10^6 PFU intraperitoneally (i.p.)) PCR for GP DNA was positive after 3 months despite clearance of culturable virus from the spleen. In contrast, 2/2 guinea pigs, rats and accidentally infected humans (>2 years) were all negative for DNA.

A sensitive assay for reverse transcriptase (RT)¹³ demonstrated high levels in murine and hamster lines and undetectable levels in Vero and HeLa cells (monkey and human, respectively); cell lines

Figure 1 Identification of LCMV-derived DNA after infection *in vivo*. **a**, PCR amplification strategy. The S strand of LCMV encodes glycoprotein and nucleoprotein encoded in opposite sense and is represented schematically here. Primers are shown consistent with the cDNA sequences for LCMV-WE strain previously published²⁹. Two sets of NP primers were used, the pairs 2818/3060 and NP5'1/NP3'1 (shaded) in a nested protocol giving rise to a product of 207 bp, and the pair A5'O/A3'O (white) using a single round of PCR giving rise to a product of 535 bp. The nested pairs 001/R1 and DM1/RC1 were used to amplify LCMV GP giving rise to a product of 907 bp; a and b represent 19-bp terminal complementary sequences common to all arenaviruses. **b**, PCR on DNA extracted from the spleens of LCMV-infected C57BL/6 mice. RNase A-treated DNA was subjected to PCR using nested primers specific for NP (see Methods), products run on 1% agarose gels, and visualized with ethidium bromide. Product size 207 bp. Spleens used in this experiment were from either uninfected mice (lanes 1, 2), mice infected 8 days previously with 200 PFU LCMV-WE i.e. (3,4), or mice infected in an identical fashion 70 days previously (5,6). **c**, Sample experiment from C57BL/6 mice infected 225 days previously with 200 PFU LCMV-WE. RNase-treated DNA from spleens of 3 mice were subjected to PCR specific for LCMV NP using the primer pair A5'O/A3'O, and products visualized as above. Product size 535 bp. Controls in this experiment were no DNA and splenic DNA from 3 uninfected mice. The positive control was cDNA template from MC57 cells infected with LCMV (M.O.I. 0.02) and cultured for 48 h. **d**, Effect of RNase and DNase treatment. DNA extracted from an immune mouse 60 days after infection with LCMV-WE was either left untreated, or subjected to a further round of RNase A treatment or DNase treatment followed by heat inactivation of the enzyme (see Methods). All samples were then subjected to nested PCR with NP-specific primers. Lane 1 is a no-DNA control. Similar treatment with RNase H failed to destroy the PCR template. **e**, Failure to amplify reverse-transcribed perforin sequences *in vivo*. Nested PCR for a splice-specific form of murine perforin cDNA was performed using primers situated in exons 2 and 3 (see Methods for PCR and cDNA synthesis)⁹. Templates used were either splenic cDNA reverse-transcribed from RNA *in vitro* using random hexamers (lane 2) or anchored poly-T primer (lane 3) or RNase-treated splenic DNA from LCMV-immune mice as in Fig. 1b,c (lanes 4,5). Lane 1 is a no-DNA control. Samples from lanes 2-5 were positive for the presence of DNA using a PCR specific for perforin exon 3 alone (see Methods).

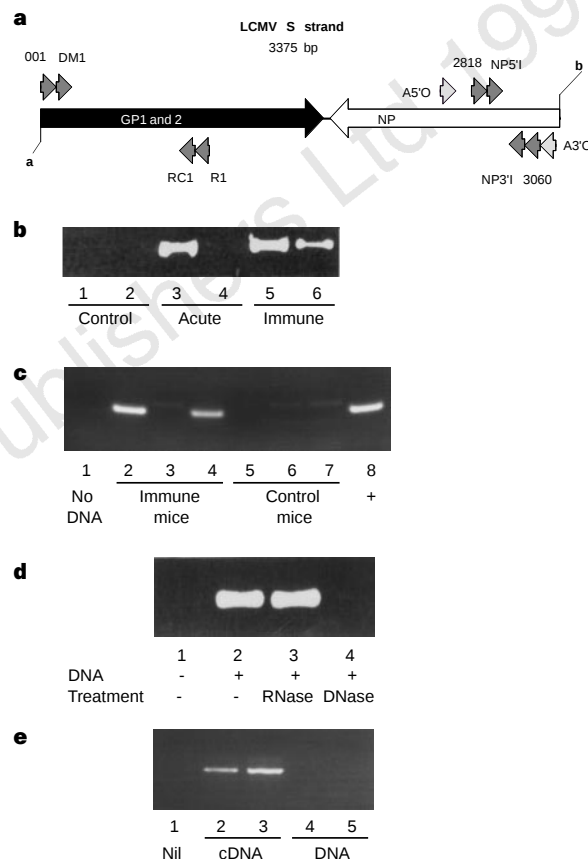
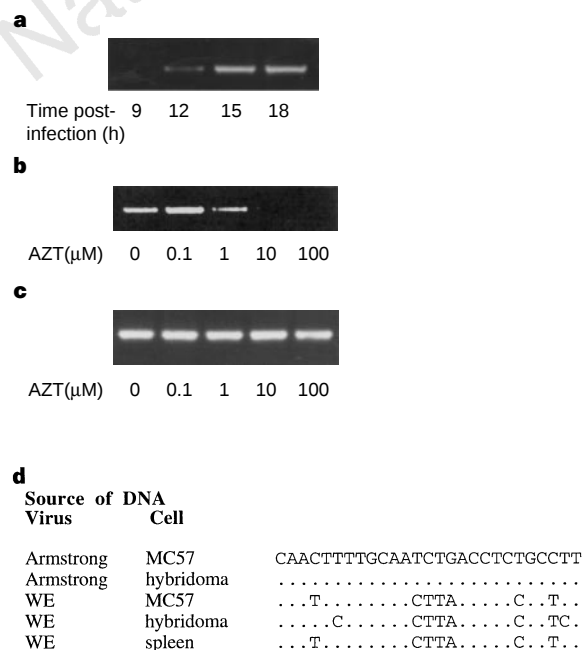


Figure 2 Analysis of LCMV DNA production *in vitro*. **a**, Time course of DNA appearance in nuclear extracts of MC57 cells. MC57 fibroblasts were infected with LCMV at multiplicity of infection (MOI) of 10 to ensure a synchronous infection, and at the time points shown, nuclear extracts obtained by centrifugation over glycerol, and then DNA prepared and PCR-amplified using GP-specific primers as described in Methods. Earlier experiments had shown DNA signal appearance in whole-cell extracts at 8–10 h, therefore the timepoints illustrated are 9, 12, 15 and 18 h. Similar time courses were also observed with lower MOI (0.1–1). **b**, **c**, Effect of AZT on production of LCMV DNA *in vitro*. **b**, Confluent cell monolayers in 24-well plates were pretreated with AZT (Sigma) at the concentrations 0.1–100 μ M for 3 h, and subsequently infected with LCMV-WE at an MOI of 10 as above. Cells were cultured in the continued presence of AZT for a further 21 h and supernatants collected and DNA extracted. Nested PCR was performed with GP-specific primers and products visualized as above. In parallel experiments, AZT treatment caused no inhibition of LCMV production *in vitro* at similar concentrations using MOI from 0.01–10 up to 48 h (peak production $5.9\text{--}7.5 \log \text{PFU ml}^{-1}$). **c**, The PCR amplification of a 320-bp product derived from genomic perforin exon 3 (see Methods for primers and conditions). **d**, Confirmation of specificity of PCR products. PCR products were amplified using GP-specific PCR and directly sequenced using primers RC1 and DM1. The sequence from the central region of GP1 bp 435–462 is shown. Sequences were obtained from DNA extracted from MC57 cells infected with either Armstrong or WE strains, or B-cell hybridomas derived *ex vivo* infected with these viruses¹¹, or immune mouse splenic tissue obtained as in Fig. 1a from an LCMV-WE-infected mouse. Although all isolates were consistent with standard sequences, the heterogeneity seen in LCMV-WE sequences from different sources renders cross-contamination within the laboratory unlikely.



from other species (dog, rat, gerbil) showed intermediate levels. This confirms the presence of endogenous RT in those lines capable of formation of a virus-specific DNA product; however, presence of RT is not sufficient to lead to generation of cDNA after infection. One guinea pig line (CRL 158) contained high levels of RT in supernatant but was unable to form LCMV-specific DNA after infection, indicating that the differences may not lie only in the level but also in the specific form of RT or other intracellular factors present. LCMV cultures derived from infection of RT-negative Vero cells were negative for RT activity, although it remains possible that the LCMV polymerase itself possesses some RT activity, which only leads to DNA formation in the presence of specific cellular factors.

Cell subsets forming LCMV-specific DNA after infection *in vivo* included peritoneal macrophages and both T-cell-depleted and -enriched fractions of splenocytes (data not shown). Bone marrow-derived cells, including peritoneal macrophages from C57BL/6, BALB/c and, notably, wild mice, as well as LCMV-infected T-cell hybridomas¹⁴ also contained LCMV-specific DNA after infection *in vitro*. Taken together, this indicates that cells that reverse-transcribe viral RNA during the course of natural infection include lymphocytes and antigen-presenting cells.

To investigate further the ability of LCMV-specific DNA to persist after disappearance of replicating virus, as *in vivo*, murine cell lines were cultured for extended periods after infection *in vitro*. After 11–15 days in murine MC57 and L cells, no culturable virus was detectable in supernatant (below a threshold of 0.7 log PFU ml⁻¹), although other cell lines continued to release virus efficiently (Fig. 3a). This disappearance of virus within a few days was associated with loss of immunofluorescence for GP and NP and was specific for LCMV-WE strain; consistent with previous studies, LCMV-Armstrong infection led to continuous low-level virus production *in vitro* (data not shown)^{15,16}. Cells from extended LCMV-WE-infected MC57 cultures were then tested for lysis by LCMV-specific CTL, which recognize epitopes in GP and NP¹⁷. Such effector T cells, which lyse acutely infected cells with nearly 100% efficiency were unable to lyse infected cells after longer periods of culture (Fig. 3b). PCR for LCMV DNA was, however, consistently positive in cultures of MC57 cells 3 weeks after infection (Fig. 3b). Limiting dilution experiments suggested about 1 copy per 10³ to 10⁴ MC57 cells. These experiments indicate not only that the reverse-transcribed product is long-lived, but also that its detection does not depend on concurrent high levels of viral turnover. Such DNA may exist extrachromosomally, possibly in circular forms as postulated for

LCMV RNA¹⁸, or potentially may integrate, although the latter event is likely to be extremely rare even compared to endogenous retrotransposed elements¹⁹.

The possibility that endogenous RT reverse-transcribes RNA derived from other infectious viruses has been previously suggested²⁰. The source of the RT could be an endogenous retrovirus, or a variety of other interspersed elements²¹; reverse transcripts from such sources may account for 10–20% of the mammalian genome²². This study provides evidence that endogenous RT can, in a host-virus-specific manner, generate cDNA transcripts from LCMV as it infects cells *in vitro* and *in vivo*, and that this cDNA persists long after the original acute infection has waned below detectable levels. LCMV infection upregulates endogenous retrovirus replication in the mouse²³. For another murine RNA virus, lactate dehydrogenase virus (LDV), such endogenous retroviral activity is actually required for successful infection of neural tissue²⁴.

Even if viral sequences should persist as RNA elsewhere in the host²⁵, the finding of virally determined DNA comes as a surprise. This observation broadens the spectrum of potential virus-host relationships (which already extends from acute cytopathic DNA

Table 1 Species-specific production of LCMV DNA

Line	Origin	Titre	RT*	LCMV DNA PCR	
				<i>in vitro</i>	<i>in vivo</i>
MC57	Mouse	6.9	>200	+	+
L 929	Mouse	7.5	>200	+	+
IC21	Mouse	5.1	>200	+	+
BHK	Hamster	4.6	>200	+	+
CCL 158	Guinea-pig	7.8	>200	–	–
CRL 1405	Guinea-pig	7.5	88	–	–
CCL 100	Gerbil	5.5	9	–	NT
208F	Rat	3.7	4	–	–
MDCK	Dog	4.8	55	–	NT
BSC 40	Cow	5.8	<0.1	–	NT
Vero	Monkey	6.1	<0.1	–	NT
Hela	Human	6.2	<0.1	–	–

In vitro experiments were performed using cells infected for 1 h at a multiplicity of infection of 0.1, washed and plated in 25-cm² tissue culture flasks. Virus supernatant was collected at 48 h and infectious virus titred by immunofocus assay⁶. DNA was extracted as previously described and PCR directed at LCMV GP performed. *In vivo* experiments were performed using DNA extracted from mouse infected as previously, hamster or guinea-pig splenic tissue 8 weeks after infection and rats 1 week after infection (200 PFU i.v. in mouse, 2 × 10⁶ PFU i.p. in hamster, guinea-pig and rat). Acute infection in guinea-pigs and hamsters was confirmed by positive blood cultures 8 days after infection. Peripheral blood mononuclear cells (PBMC) in two laboratory workers showing neutralizing antibody responses against LCMV (and therefore presumptively infected, because only replicating virus is capable of neutralizing antibody induction) was also tested. Extracted DNA was amplified using GP-specific PCR.

* RT assays by product-enhanced reverse transcription (PERT), a PCR-based assay in which bacteriophage MS2 RNA acts as an initial template for cDNA synthesis^{13,30}. Figures represent μ units μ l⁻¹ RT equivalent.

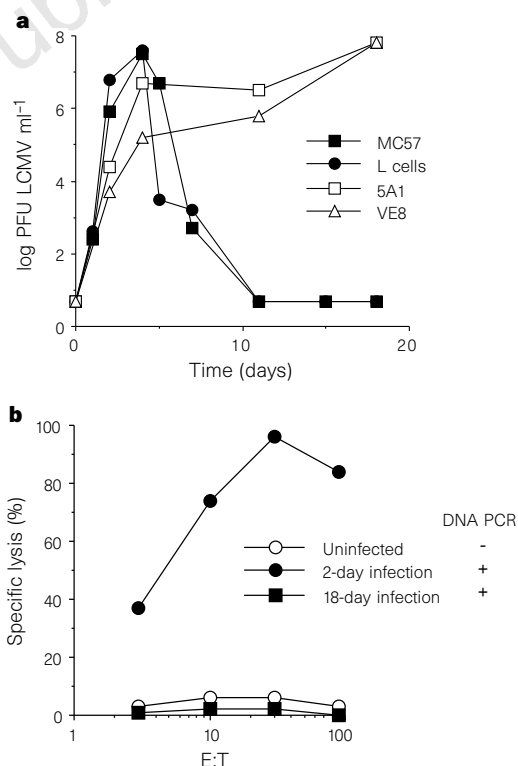


Figure 3 Persistence of LCMV-specific DNA in cells after acute infection. **a**, Time course of virus production *in vitro* after infection of cell lines. Cell lines MC57, L 929 and 5A1 and VE8 T-cell hybridomas were infected at day 0 with LCMV-WE (MOI 0.02) and cultured over the time period shown. Supernatant was collected for virus at the time points indicated and replicative virus titred by immunofocus assay. The lower limit of detection in this assay is 0.7 log PFU ml⁻¹ as seen at time-point zero. Some infected T-cell hybridomas were also found to lose virus production after prolonged culture for 6–8 weeks. **b**, CTL lysis of MC57 cells early and late after infection with LCMV. Cells were infected and cultured as above and used as targets in a chromium release assay after 2 days or 18 days of culture. Effectors were splenocytes derived from mice infected 8 days previously with LCMV-WE 200 PFU i.v. The mean values of 3 mice are shown. PCR for LCMV NP was also performed as above using RNase-treated DNA from uninfected MC57 cells, acutely infected MC57 cells, or MC57 cells 19 days after infection. These cells were negative by immunofluorescence using monoclonal antibodies directed against both GP and NP¹¹. E : T, effector : target ratio.

viruses, through those with an integral step of retrotranscription such as hepatitis B virus, to complete retroviral elements) to include non-retroviral RNA viruses in which host-derived and host-specific retrotranscription can occur. It may also be relevant for human persistent RNA viruses such as hepatitis C virus and measles²⁶.

The functional role of the LCMV DNA produced by endogenous RT activity remains to be explored. It is possible that it represents merely a gravestone marking a previous high level of infection: alternatively, complete infectious virions may be reconstituted. Between these extremes there might be sufficient levels of transcription to generate low levels of viral peptide for presentation to T cells. The ability of antigen-presenting cells such as macrophages and B cells to accumulate viral DNA genome thus potentially represents a naturally produced form of DNA vaccine²⁷. In this context even low levels of major histocompatibility complex (MHC) peptide complexes on a few cells in lymphoid organs may be immunologically important²⁸. The mechanisms involved in the maintenance of immunological memory may be multiple, but persistence of viral template in stable DNA form may contribute to this. □

Methods

Polymerase chain reaction. Mice were infected with 200 PFU LCMV (WE strain) intravenously and spleens removed at day 60–225. DNA was extracted from spleens using the Quiamp Tissue Kit (Qiagen) including an RNase A step (2 mg ml⁻¹, 30 min, room temperature). Further RNase treatment was also performed for 30 min at room temperature in the presence of 2 mg ml⁻¹ RNase A. Treatment with DNase I (20 units per 10 µl reaction volume; Boehringer Mannheim) or RNase H (1 unit per 10 µl reaction volume; Amersham) was performed at 37°C for at least 90–120 min. DNA (1 µg) was used in PCR reactions. All amplifications were performed in the presence of 0.2 µM dNTPs, 1 unit Taq polymerase (Boehringer Mannheim) and 1× PCR buffer containing 1.5 mM MgCl₂ in a total volume of 50 µl. PCR primers were present at 20 pmol per reaction. For GP PCR the primers were 001 and R1 for the primary reaction, DM1 and RC1 for the secondary. The conditions were 94°C for 1 min, 55°C for 1 min, 72°C for 2 min, 35 cycles (primary), 94°C for 1 min, 42°C for 1 min and 72°C for 1 min, 35 cycles (secondary). In the secondary reaction 1 µl of each primary product was used. For nested NP PCR the primers were 2818 and 3060 for the primary and NP5'I and NP3'I for the secondary reaction; conditions were 94°C for 30 s, 50°C for 1 min, 72°C for 30 s, 35 cycles for both amplifications.

For single-round PCR, Taq Gold (Perkin Elmer) was used in the presence of 3 mM MgCl₂ and primers A5'O and A3'O; the other components, volume and cycling conditions were as for NP nested PCR above, with the addition of an initial 10-min activation step.

For PCR specific for the spliced form of perforin⁸, primary primers were P30 (exon 2)/P33 (exon 3), secondary primers P30/P20 (exon 3), with the other components as above. Cycling conditions were 94°C for 30 s, 55°C for 30 s, 72°C for 1 min, 35 cycles for both amplifications. For the perforin genomic segment, primer pairs P20/P21 (both from exon 3) were used, with the same cycling conditions. Positive controls for the PCR specific to spliced form were cDNAs reverse-transcribed using a poly-T primer or random hexamers (First Strand Synthesis kit; Amersham), with template RNA derived from BALB/c mice infected 7 days previously with 200 PFU LCMV-WE i.v.

Primers (5'–3') 001-CGCACCGGGGATCCTAGGCTT
R1-TCGTAGCATGTACAG
DM1-GAATTCTATCCAGTAAAGGATGG
RC1-GAGCTCTGCAGCAAGGATCATCC
2818-GATGAATTCTTCACATCCAAACCC,
3060-GATCCGAATTCTACATCAAAG
NP5'I-TACTACACCACTTGCACCCTG
NP3'I-TCTGAAAGTGGAAGACTTAG.
A5'O-AAGCCAAGGTCTGTGAG
A3'O-GTTACAGAGGATCATGAG
P30-AGGCAGCTGCTAATATCAAT
P33-CCGGGGATTGTTATTGTTCC
P20-CCGGTCTGAACTCCTGGCCAC
P21-CCCCTGCACACATTACTGGAAG

To minimize the risks of contamination, template preparation, PCR mix preparation, and addition of DNA to the mix were performed in separate locations and using aerosol-resistant tips. One water control was performed per primary and secondary reaction and was uniformly negative. Cloned DNA was handled and stored in a separate laboratory. PCR reactions were also performed using independently purified DNA and samples tested independently to confirm results.

Virus culture and cell lines. Detection of replicating LCMV in blood, tissue and tissue culture supernatant by immunological focus assay has been previously described⁶. Lines CCL 100, CCL 158 and CRL1405 were obtained from the ATCC (Rockville, Maryland); T-cell hybridomas 5A1 and VE8 were obtained from LCMV-infected mice as previously described¹⁴. Other cell lines were grown under standard laboratory conditions and were free from LCMV infection before use.

CTL assays. The assays were performed exactly as previously described⁷ by detection of release of ⁵¹Cr from labelled target cells in the presence of splenocytes from C57BL/6 mice 8 days after intravenous infection with LCMV-WE at 200 PFU.

Received 2 May; accepted 9 September 1997.

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Acknowledgements. This work was supported by the Wellcome Trust, the Swiss National Foundation for Science and the Kanton of Zurich, Switzerland. We thank E. Horvath, H. Krettl, A. Oxenius, S. Oehen, K. Riem, O. Planz, P. Seiler, N. Wey, D. Zimmerman and his group and J. Schupbach and J. Boeni of the Swiss National Centre for Retroviruses, University of Zurich for their technical help; we also thank L. Stitz (BFAV, Tübingen) and J. Klein (MPI, Tübingen) for providing mice and D. Bishop, J. Goudsmit, M. Billeter and R. Cattaneo for helpful discussions.

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Regulation of CFTR chloride channels by syntaxin and Munc18 isoforms

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The cystic fibrosis gene encodes a cyclic AMP-gated chloride channel (CFTR) that mediates electrolyte transport across the luminal surfaces of a variety of epithelial cells^{1–4}. The molecular mechanisms that modulate CFTR activity in epithelial tissues are poorly understood. Here we show that CFTR is regulated by an epithelially expressed syntaxin (syntaxin 1A), a membrane protein that also modulates neurosecretion^{5–7} and calcium-channel gating^{8–11} in brain. Syntaxin 1A physically interacts with CFTR chloride channels and regulates CFTR-mediated currents both in *Xenopus* oocytes and in epithelial cells that normally express these proteins. The physical and functional interactions between syntaxin 1A and CFTR are blocked by a syntaxin-binding protein of the Munc18 protein family (also called n-Sec1; refs 12–14). Our

results indicate that CFTR function in epithelial cells is regulated by an interplay between syntaxin and Munc18 isoforms.

Figure 1 shows that syntaxin 1A and a Munc18 isoform are detectably expressed in human colonic epithelial cells. In initial immunoblotting experiments, we detected a 35K (M_r 35,000) membrane protein in T84 and HT29-CL19A human colonic epithelial cell lines that was recognized by a panel of syntaxin 1-specific antibodies (Fig. 1a, b). We confirmed that colonic epithelial cells express the complementary DNA encoding human syntaxin 1A by screening a T84 cDNA library at low stringency using a rat syntaxin 1A cDNA probe (results not shown). During the screening of this epithelial library, we also isolated the cDNA encoding the human isoform of another member of this protein family, syntaxin 3 (Genbank accession number U32315). By quantitative immunoblotting, we determined that syntaxin 3, like syntaxin 1A, is expressed in human colonic epithelial cell lines, as well as in native colon and trachea, and at 5–10 times higher levels than syntaxin 1A (results not shown). Multiple antibodies to each isoform labelled the apical poles of T84 and HT29-CL19A colonic epithelial cells in immunofluorescence experiments (results not shown). We also affinity-purified a syntaxin 1A-binding protein from T84 cells that was determined to be a Munc18 isoform on the basis of microsequencing (Fig. 1c) and immunoblotting (data not shown). Thus, colonic epithelial cells express syntaxins 1A and 3 and a high-affinity syntaxin-binding protein of the Munc18 family^{12–14}.

CFTR chloride channels interact with syntaxin 1A in an isoform-specific manner (Fig. 2). CFTR could be precipitated from colonic epithelial cell lysates with a glutathione-S-transferase (GST) fusion protein containing the cytosolic domain of syntaxin 1A (GST-syn1A Δ C, where Δ C refers to deletion of the C-terminal membrane domain⁵). No such binding was observed for six other proteins that are more abundant in epithelial cells than CFTR, including the

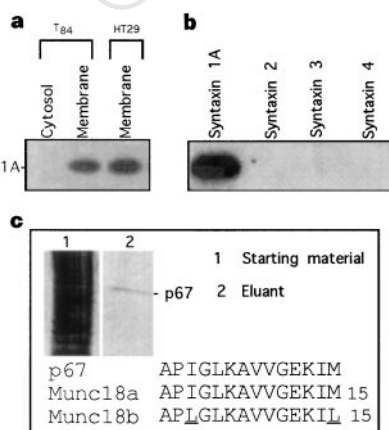


Figure 1 Expression of syntaxin and Munc18 isoforms in colonic epithelial cells. **a**, Immunoblot detection of syntaxin 1 in crude membranes prepared from T84 and HT29-CL19A colonic epithelial cell lines (50 μ g each lane). Membranes and cytosol were prepared as described²⁶. **b**, Isoform specificity of syntaxin 1 polyclonal antibody verified by immunoblotting recombinant cytosolic domains of the indicated syntaxins (50 ng each lane). The 35K membrane protein in colonic cells was also detected using two additional syntaxin 1-specific polyclonal antibodies and a commercially available syntaxin 1-specific monoclonal antibody (HPC-1; Sigma; results not shown). **c**, Purification and microsequence of Munc18 isoform from T84 colonic epithelial cells. Shown are Coomassie-blue-stained starting material (crude membrane extract; 50 μ g) and 67K protein eluted from a GST-syn1A Δ C affinity column in 1M NaCl. Purification method has been described¹².

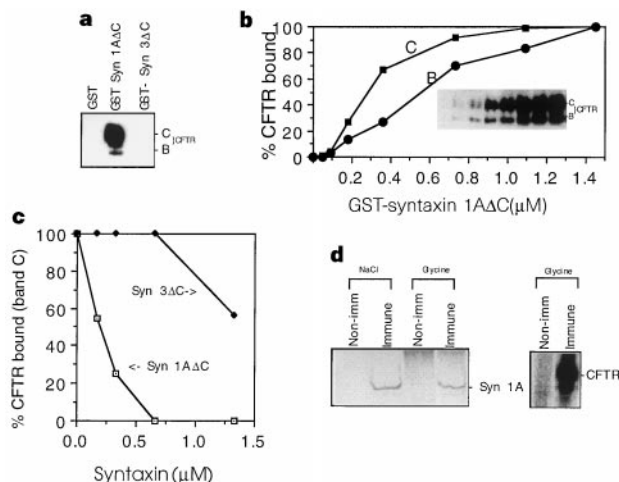


Figure 2 CFTR physically interacts with syntaxin 1A. **a**, *In vitro* binding of CFTR in HT29-CL19A cell lysates to recombinant cytosolic domain of syntaxin 1A fused to GST (GST-syn1A Δ C) and immobilized on glutathione-agarose beads (50 μ g each fusion protein). Bound CFTR was detected by immunoblotting. Bands B and C represent immature and mature CFTR, respectively. **b**, CFTR binding saturates at submicromolar concentrations of soluble GST-syn1A Δ C. Immunoblot of CFTR binding at each GST-syn1A Δ C concentration is shown in inset (repeated three times with similar results). **c**, Competition for CFTR binding between GST-syn1A Δ C (10 μ g throughout) and increasing concentrations of syntaxin cytosolic domain cleaved free of GST by thrombin (repeated 3 times with similar results). **d**, Syntaxin 1 co-immunoprecipitates with CFTR from HT29-CL19A cell lysates. Proteins immunoprecipitated with CFTR antibody were eluted in 1.0M NaCl followed by 0.1 M glycine (pH 2.5) and probed for syntaxin 1A by immunoblotting and for CFTR by immunoprecipitation followed by *in vitro* phosphorylation as described²⁰.

apical membrane marker aminopeptidase (results not shown). Both the mature form (band 'C') and immature form (band 'B') of CFTR² exhibited syntaxin 1A-binding activity, although the affinity of this interaction is higher for the mature form (Fig. 2b). In solution binding assays, we determined that the binding of CFTR to syntaxin 1A was saturable with half-maximal binding of band 'C' at a GST-syn1AΔC concentration of ~300 nM. The binding of CFTR to GST-syn1AΔC could be competitively inhibited by submicromolar concentrations of soluble syntaxin 1A that had been cleaved free of GST (Fig. 2c). Soluble syntaxin 3 cytoplasmic domain was less effective at inhibiting the interaction between CFTR and GST-syn1AΔC (Fig. 2c), which confirms the isoform specificity of this interaction (see also Fig. 2a). We were also able to co-immunoprecipitate syntaxin 1A with CFTR from colonic epithelial cell lysates (Fig. 2d) and from COS-7 fibroblasts expressing recombinant CFTR and syntaxin 1A (results not shown) using multiple antibodies raised against each protein. However, the efficiency with which

these two proteins could be co-immunoprecipitated from detergent lysates was low, consistent with the dynamic nature of the functional interactions between these proteins (see below).

We examined the ability of recombinant syntaxin 1A to regulate CFTR-mediated chloride currents in two-electrode voltage-clamp studies of *Xenopus* oocytes (Fig. 3). CFTR currents in cRNA-injected oocytes exhibited linear current-voltage behaviour and were blocked by diphenylamine carboxylate (results not shown), as described^{15,16}. Full-length syntaxin 1A inhibited CFTR-mediated currents (Fig. 3a) in a dose-dependent manner (Fig. 3b) without changing their linear I-V behaviour or reversal potential (not shown). The syntaxin 1A inhibition of CFTR currents was acutely reversed by injection of botulinum neurotoxin C1 (BONT/C1; Fig. 3c), an endoprotease that cleaves the cytosolic domain of syntaxin 1A from its membrane anchor⁶. BONT/C1 had no effect on CFTR currents in the absence of syntaxin 1A (Fig. 3c). In contrast to syntaxin 1A, syntaxin 3 failed to inhibit CFTR currents and

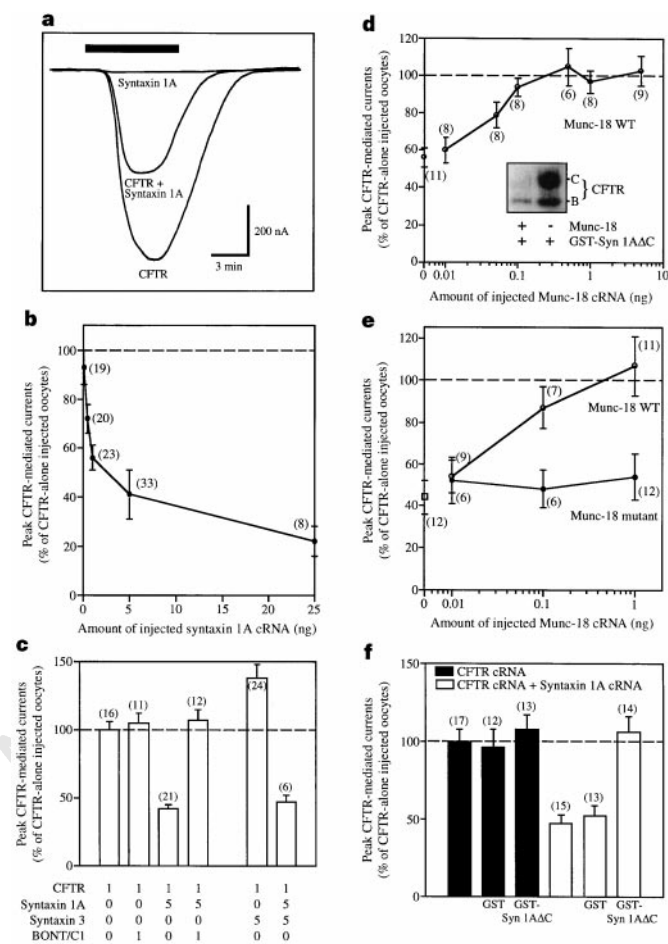


Figure 3 Syntaxin 1A and Munc18 regulate CFTR-mediated chloride currents in *Xenopus* oocytes. **a**, CFTR-mediated chloride currents in oocytes injected with 1 ng CFTR cRNA, 5 ng syntaxin 1A cRNA, or both. The bar above the traces indicates the duration of application of cAMP cocktail (see Methods). **b**, Dose-dependent inhibition of CFTR currents by syntaxin 1A. **c**, Injection of botulinum neurotoxin C1 (1 ng per oocyte) 30–60 min before current recording reverses the syntaxin inhibition of CFTR, whereas syntaxin 3 does not inhibit CFTR currents. **d**, Munc18a rescues CFTR from syntaxin 1A inhibition in oocytes and blocks the *in vitro* binding of CFTR to GST-syn1AΔC (inset). Bands B and C represent immature and mature CFTR, respectively. **e**, Co-expression of a Munc18a mutant (D34N; M38V) that lacks syntaxin-binding activity does not prevent syntaxin 1A inhibition of CFTR currents. **f**, Injection of GST-syn1AΔC (estimated final concentration, 50 μg ml⁻¹) but not GST (50 μg ml⁻¹) 40–60 min before current recording reverses the inhibition of CFTR currents by full-length syntaxin 1A. Numbers of oocytes assayed are shown in parentheses.

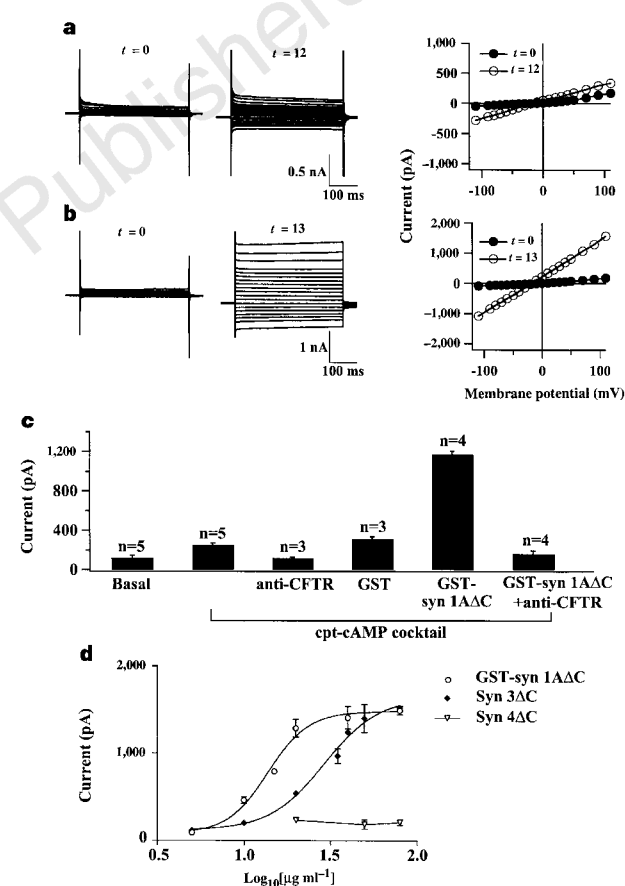


Figure 4 Soluble syntaxin 1A cytosolic domain potentiates CFTR-mediated chloride currents in T₈₄ colonic epithelial cells that endogenously express CFTR and membrane-anchored syntaxin 1A. **a**, Left: basal whole cell currents recorded immediately after establishing whole-cell configuration in the presence of 500 μM cpt-cAMP in the pipette solution; centre, currents recorded 12 min later; right, associated current-voltage relationship (I-V) at t = 0 min (black circles) and 12 min (white circles). **b**, Whole-cell currents from a cell voltage-clamped as in **a**, with the addition of 350 nM GST-syn1AΔC (20 μg ml⁻¹) to the pipette solution. **c**, Summary of mean peak current amplitude for data obtained from T₈₄ cells at a holding potential of +110 mV following a 40-min equilibration with a pipette solution that contained GST (750 nM), GST-syn1AΔC (350 nM), and/or CFTR antibody (0.1 μg ml⁻¹). Currents were activated with a cAMP cocktail added to the extracellular bath (see Methods). Data are expressed as mean ± s.e.m. **d**, Isoform specificity of the potentiation of epithelial CFTR chloride currents by syntaxin cytosolic domains. The syntaxin-dependent increases in Cl⁻ current measured at +110 mV in the presence of the cAMP cocktail are shown (n = 3–7 cells for each data point).

instead had a moderate stimulatory effect which was overcome by co-expression with syntaxin 1A. Inhibition of CFTR currents by syntaxin 1A was CFTR-specific; that is, syntaxin 1A did not inhibit the activities of either the α -7 homomeric nicotinic acetylcholine receptor or the GAT1 GABA transporter when these molecules were expressed in oocytes (results not shown).

CFTR currents in oocytes could be rescued from syntaxin 1A inhibition by two additional manoeuvres: co-expressing recombinant Munc18a or by injecting the soluble cytosolic domain of syntaxin 1A (Fig. 3d, e, f). Figure 3d shows that Munc18a inhibited the *in vitro* binding of syntaxin 1A to CFTR (inset) and prevented the inhibition of CFTR currents by syntaxin 1A in a dose-dependent manner when these molecules were co-expressed in oocytes. A Munc18a mutant that lacks syntaxin-binding activity (binding data not shown) was unable to rescue CFTR currents from syntaxin inhibition (Fig. 3d). Figure 3f shows that the cytosolic domain of syntaxin 1A (GST-syn1A Δ C), like BONT/C1, also acutely reversed the inhibition of CFTR currents by full-length syntaxin 1A when injected into co-expressing oocytes. GST-syn1A Δ C had no effect on CFTR currents in the absence of full-length syntaxin 1A, so this soluble fragment stimulated CFTR currents by disrupting the functional interaction between CFTR and membrane-anchored syntaxin 1A. Together with our BONT/C1 data, these results indicate that: (1) syntaxin 1A must be membrane-anchored to inhibit CFTR currents, as also suggested for the regulation of calcium channels by syntaxin 1A (ref. 9), and (2) the interaction

between CFTR and membrane-anchored syntaxin 1A is dynamic (that is, acutely reversible).

We next examined the biological relevance of the interactions among CFTR, syntaxin 1A and Munc18 by performing whole-cell patch-clamp studies of epithelial cells that endogenously express these three proteins. Figure 4 shows that the cytoplasmic domain of syntaxin 1A (GST-syn1A Δ C) augments CFTR currents in colonic epithelial cells, as it should if endogenous membrane-anchored syntaxin 1A normally attenuates the activity of native CFTR in these cells. Inclusion of 350 nM GST-syn1A Δ C in the patch pipette resulted in a 2–3-fold stimulation of whole-cell chloride currents activated by a maximal dose of cAMP in T84 cells (Fig. 4) or Caco-2 colonic epithelial cells (data not shown). These large currents appeared within 2–5 min of membrane rupture by the patch pipette and were CFTR-mediated on the basis of three criteria: voltage-independent with a chloride-selective reversal potential (Fig. 4a, b; refs 15, 16); insensitive to 1 mM DIDS in the extracellular bath but inhibited $90 \pm 1\%$ ($n = 3$) by 1 mM diphenylamine carboxylate^{17,18}; and inhibited by a CFTR-blocking antibody^{19,20} (Fig. 4c). GST alone had no effect on cAMP-activated chloride currents (Fig. 4c). In addition, GST-syn1A Δ C had no significant effect on currents that were measured in the absence of cAMP or on currents that were activated by calmodulin-dependent kinase II (results not shown), which modulates a non-CFTR-mediated chloride conductance in these cells^{21,22}. The potentiation of epithelial CFTR currents by soluble syntaxin was isoform-specific, with a rank order of syntaxin 1A $\gg$ 3 $\gg$ 4 (Fig. 4d). The half-maximal effective concentration (EC_{50}) for CFTR current activation by GST-syn1A Δ C (~ 250 nM) agreed well with the EC_{50} for *in vitro* binding (~ 300 nM; Fig. 2b). The ability of GST-syn3A Δ C to activate CFTR currents at higher concentrations is presumably due in part to its ability to compete weakly with endogenous syntaxin 1A for CFTR binding (Fig. 2c), although this construct may influence CFTR activity in other ways as well (by altering apical membrane traffic, for example).

Figure 5 shows that epithelial CFTR currents are also potentiated by two other reagents that rescued CFTR from inhibition by membrane-anchored syntaxin 1A in oocytes: BONT/C1 and Munc18a. Microinjection of BONT/C1 into T84 colonic epithelial cells potentiated cAMP-activated whole-cell currents to the same degree as a maximal dose of GST-syn1A Δ C (Fig. 5a). The neurotoxin had no significant effect on basal chloride currents nor did the toxin have an additive effect on cAMP-induced currents when combined with GST-syn1A Δ C, as would be expected if these reagents activate CFTR currents by the same mechanism. Recombinant Munc18a also stimulated CFTR-mediated chloride currents in a dose-dependent fashion when introduced through the patch pipette (Fig. 5b), which indicates that this soluble syntaxin-binding protein is a limiting factor in the activation of 'whole cell' CFTR currents in these epithelial cells. In addition, at very low concentrations, Munc18a blocked the effect of GST-syn1A Δ C on CFTR currents, presumably by inhibiting the binding of soluble syntaxin 1A to CFTR. Thus, Munc18a also modulates CFTR function in epithelial cells probably by controlling the availability of syntaxin 1A for CFTR.

In summary, our results indicate that CFTR physically and functionally interacts with an epithelially expressed syntaxin 1 isoform that is a negative modulator of CFTR-mediated chloride currents. This negative modulation of CFTR function by syntaxin 1A occurs in epithelial cells as well as in heterologous expression systems, because epithelial CFTR currents were markedly potentiated by three different reagents that rescue CFTR from inhibition by membrane-anchored syntaxin 1A in oocytes (that is, BONT/C1, Munc18 and syntaxin 1A cytosolic domain). The mechanism by which syntaxin 1A modulates CFTR currents could involve regulation of CFTR traffic to or from the cell surface or direct regulation of CFTR channel activation through protein–protein interactions. This latter mechanism is consistent with the emerging view that

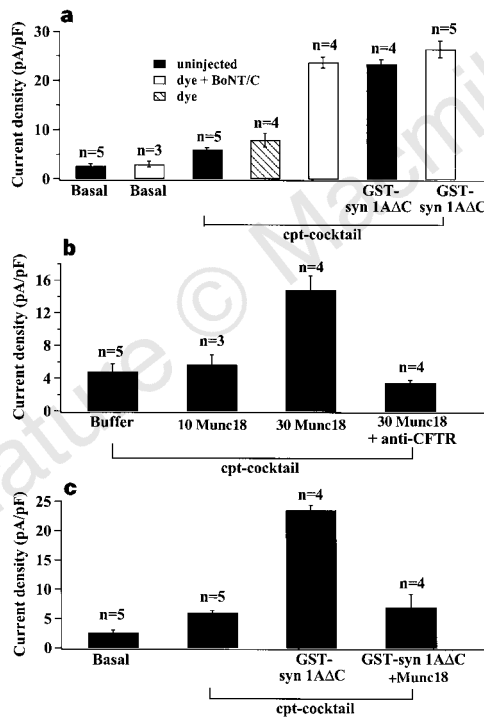


Figure 5 Botulinum neurotoxin C1 and Munc18a also potentiate CFTR-mediated currents in epithelial cells. **a**, BONT/C1 mimics the effects of soluble GST-syn1A Δ C on cAMP-activated Cl^- currents in epithelial cells. T84 cells were microinjected with 0.5% Texas red for later identification, or with dye plus 1 ng μl^{-1} BONT/C1 6–24 h before patch clamping. Cl^- currents were activated with the cAMP cocktail added to the bath (see Methods) in the presence or absence of 350 nM GST-syn1A Δ C in the pipette. **b**, GST-Munc18a fusion protein augments CFTR-mediated currents in T84 cells in a concentration-dependent manner. Comparison of mean peak Cl^- currents under control conditions and in the presence of 10 $\mu g ml^{-1}$ GST-Munc18a, 30 $\mu g ml^{-1}$ GST-Munc18a (~ 300 nM) or 30 $\mu g ml^{-1}$ GST-Munc18a plus CFTR-blocking antibody, in the pipette. **c**, Munc18 negates the effects of GST-syn1A Δ C on CFTR currents in T84 cells. Comparison of mean peak Cl^- currents under control conditions and in the presence of 20 $\mu g ml^{-1}$ GST-syn1A Δ C with or without 10 $\mu g ml^{-1}$ GST-Munc18a in the pipette.

membrane-anchored syntaxin 1A directly modulates N-type calcium-channel activity in brain^{9–11}. It will be important to determine whether syntaxin 1A directly binds to CFTR and if it regulates epithelial CFTR chloride channels and neuronal calcium channels by similar mechanisms. We speculate that the CFTR–syntaxin–Munc18 interactions play a role in fine-tuning CFTR activity in response to certain physiological cues, such as the activation of second messenger pathways that regulate the physical interactions between these proteins^{23,24}. Understanding the mechanisms by which these molecules regulate CFTR activity may be relevant to the design of strategies for augmenting epithelial CFTR function in cystic fibrosis. □

Methods

In vitro binding and co-immunoprecipitation. Qualitative 'pull down' assays were done by adding immobilized GST, GST–syn1AΔC or GST–syn3ΔC (50 μg) to a 1% NP-40 lysate of HT29-CL19A cells for 12 h at 4°C. Beads were pelleted, washed extensively in lysis buffer and analysed for CFTR by immunoblotting. Inhibition of CFTR-syntaxin 1A binding by Munc18a (Fig. 3d) was verified by preincubating GST–syn1AΔC (15 μg) with or without excess Munc18a before assaying CFTR binding. Quantitative solution binding assays were done by adding soluble eluted GST fusion protein to an HT29-CL19A cell lysate for 3 h at 4°C. GST fusion protein was then precipitated with excess glutathione–agarose and CFTR binding quantified by immunoblotting and densitometry. Co-immunoprecipitation was performed by passing HT29-CL19A cell lysates (0.8% Triton X-100 in HEPES-buffered saline (pH 7.4)) through a hydrazide-derivatized disc (Actidisc, FMC Corp) to which anti-CFTR IgG (anti NBD1; residues 426–588; ref. 20) or non-immune IgG was covalently bound. Bound proteins were eluted and analysed (Fig. 2 legend).

Electrophysiology. *Xenopus* oocytes were injected with the cRNAs encoding CFTR (1 ng), full-length syntaxin 1A (5 ng, or as indicated in Fig. 3) and Munc18a (as indicated in Fig. 3) and assayed 48–72 h later for CFTR currents²⁵. Currents were typically activated with a cocktail containing 20 μM forskolin, 100 μM dibutyl cyclic AMP and 100 μM IBMX. A similar inhibition of CFTR currents by syntaxin 1A was also observed using a supermaximal CFTR activation cocktail (20 μM forskolin, 200 μM dibutyl cAMP and 5 mM IBMX; data not shown). Whole-cell patch-clamping of T84 cells was done as described^{19,22}, except that 5 mM instead of 1 mM Mg-ATP was used in the pipette solution. Cell capacitance was measured by integrating the current during a 10-mV voltage step and subtracting a baseline established ~15 ms after the step. Currents were activated with 500 μM cpt-cAMP in the pipette or with an extracellular cocktail (100 μM cpt-cAMP, 50 μM forskolin and 50 μM IBMX) for those experiments involving pre-equilibration of the cell interior with CFTR-blocking antibody¹⁹.

Received 2 July; accepted 28 August 1997.

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Acknowledgements. We thank S. Reddy, U. Gopalakrishnan, P. St John and M. N. Shelton for assistance, E. Weber, T. Jilling, T. Elton and D. Abrahamson for advice, and T. Südhof for syntaxin 1A antibody and rat syntaxin 1A cDNA.

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Caenorhabditis elegans CED-9 protein is a bifunctional cell-death inhibitor

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The *Caenorhabditis elegans* gene *ced-9* prevents cells from undergoing programmed cell death and encodes a protein similar to the mammalian cell-death inhibitor Bcl-2 (refs 1–7). We show here that the CED-9 protein is a substrate for the *C. elegans* cell-death protease CED-3 (refs 8, 9), which is a member of a family of cysteine proteases first defined by CED-3 and human interleukin-1β converting enzyme (ICE)^{10–12}. CED-9 can be cleaved by CED-3 at two sites near its amino terminus, and the presence of at least one of these sites is important for complete protection by CED-9 against cell death. Cleavage of CED-9 by CED-3 generates a carboxy-terminal product that resembles Bcl-2 in sequence and in function. Bcl-2 and the baculovirus protein p35, which inhibits cell death in different species through a mechanism that depends on the presence of its cleavage site for the CED-3/ICE family of proteases^{9,13–17}, inhibit cell death additively in *C. elegans*. Our results indicate that CED-9 prevents programmed cell death in *C. elegans* through two distinct mechanisms: first, CED-9 may, by analogy with p35 (refs 9, 17), directly inhibit the CED-3 protease by an interaction involving the CED-3 cleavage sites in CED-9; second, CED-9 may directly or indirectly inhibit CED-3 by means of a protective mechanism similar to that used by mammalian Bcl-2.

Baculovirus p35 protein, a general inhibitor of programmed cell death^{9,13–17}, is a substrate for the *C. elegans* cell-death protease CED-3 and may act as a competitive inhibitor of CED-3 (ref. 9). We tested whether CED-9 protein, an endogenous *C. elegans* cell-death inhibitor^{1,2}, might act similarly. We found that ³⁵S-methionine-labelled CED-9 synthesized *in vitro* was cleaved by purified CED-3 protease¹⁸ to generate two products of relative molecular masses

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(M_r) 7K and 24K (Fig. 1a, lanes 1 and 2). A C-terminal truncated CED-9 fusion protein, His₁₀CED-9(1–251), in which the CED-9 putative membrane-anchoring region (amino acids 252 to 280)² was deleted, was also cleaved by CED-3 protease, yielding two major products of 22K and 9K and one minor product of 24K (Fig. 1b). The N-terminal sequences of the 22K and 24K His₁₀CED-9(1–251) cleavage products (LPSPS and GKINDW in single-letter code) indicated that CED-3 cleaved CED-9 at two different sites, between aspartate 47 and leucine 48, and between aspartate 67 and glycine 68, respectively (data not shown). The amino-acid sequences N-terminal to these cleavage sites (P_4 to P_1 ; DAQD and ESID) are similar to the consensus CED-3 cleavage site D/EXXD, in which the P_4 residue is aspartate or glutamate and X may be any amino acid (D.X. and H.R.H., unpublished observations)¹⁸. The P_4 and P_1 residues are the most important sites for substrate recognition and for the specificity of the CED-3/ICE-family proteases¹⁹.

We introduced mutations at either the P_4 (D44A) or P_1 (D67E) residue of the CED-3 cleavage sites and tested the effects of these mutations on the cleavage of CED-9 by the CED-3 protease *in vitro*. CED-9 proteins carrying mutations at either CED-3 cleavage site (D44A or D67E) were still cleaved by the CED-3 protease (Fig. 1a, lanes 3–6). CED-9 proteins with mutations at both CED-3 cleavage sites were cleaved less effectively (D44A, D67E) or hardly at all (D47E, D67E) by the CED-3 protease (Fig. 1a, lanes 7–10). The cleavage of CED-9(D67E) by CED-3 generated a 27K product instead of the 24K product seen with wild-type CED-9 and with CED-9(D44A); this 27K protein is presumably the C-terminal product generated by cleavage at the first CED-3 site, D47 (Fig. 1a).

We next investigated whether the presence of the two CED-3 cleavage sites in CED-9 is important for the ability of CED-9 to inhibit cell death in *C. elegans* by using an *in vivo* cell-death protection assay^{2,9}. Briefly, we generated transgenic nematodes expressing either wild-type or mutant CED-9 proteins under the control of the *C. elegans* heat-shock promoters, and then determined the extent of protection from cell death conferred by the expression of these proteins by counting the number of extra or 'undead' cells in the anterior pharynx of the transgenic worms after heat-shock treatment. We found that mutations that disrupted either the first CED-3 cleavage site (D44A) or the second CED-3 cleavage site (D67E) in CED-9 seemed slightly to reduce the cell-death protective function of CED-9 (Table 1). By contrast, mutations that disrupted both CED-3 cleavage sites ((D44A, D67E) or (D47E, D67E)) reduced the protective activity of CED-9 by about two-thirds. This reduction in CED-9 activity was not caused by instability of CED-9 protein resulting from the amino-acid substitutions, because these CED-9 mutant proteins were present in amounts similar to that of the wild-type CED-9 protein, as judged by western-blot analysis (data not shown). These results indicate that the presence of at least one CED-3 cleavage site in CED-9 is important for full CED-9 function in cell-death protection in *C. elegans*. We also found that the two CED-3 cleavage sites are necessary for the *ced-9* gene to rescue the *ced-9* loss-of-function mutant phenotype² (data not shown).

These experiments indicate that the mutant proteins CED-9(D44A, D67E) and CED-9(D47E, D67E) both retained significant CED-9 activity (Table 1), which was probably a consequence of the C-terminal region of CED-9, as expression of the CED-9 C-terminal cleavage product CED-9(68–280) inhibited cell death to an extent comparable to that caused by CED-9(D47E, D67E). By contrast, expression of the CED-9 N-terminal region CED-9(1–67) did not inhibit cell death (Table 1); this failure correlated with its lack of cleavage by CED-3 *in vitro* (data not shown). Co-expression of CED-9(1–67) and CED-9(68–280) protected against cell death to the same extent as did expression of CED-9(68–280) on its own (Table 1), indicating that co-expression of these polypeptides could not restore full CED-9 activity.

CED-9(68–280) is similar in sequence to the mammalian cell-

survival factor Bcl-2, especially in the two Bcl-2 homology domains known as BH1 and BH2 (Fig. 1)². Bcl-2 inhibits cell death in *C. elegans* to the same extent as does CED-9(68–280) (Table 2)^{2,20}; also, co-expression of Bcl-2 and CED-9(68–280) did not protect against cell death in *C. elegans* any better than either protein expressed by itself (Table 2), indicating that CED-9(68–280) and Bcl-2 may inhibit cell death by a similar mechanism. Thus, CED-9(68–280) and Bcl-2 may have similarity in function as well as in sequence.

To assess the contribution of the BH1 and BH2 domains to the protective effect of CED-9(68–280) against cell death, we introduced mutations into these regions. A single mutant (W214A) altered in the highly conserved Trp 214 residue of the BH2 domain of CED-9 partially reduced CED-9(68–280) activity (Table 2). A double mutant (G169L, W214A) that was additionally altered at a highly conserved glycine 169 residue in the BH1 domain abolished the protective activity of CED-9(68–280) (Table 2). Expression of both CED-9(68–280) mutant proteins was comparable to that of the original CED-9(68–280) (data not shown). These results indicate that both the BH1 and BH2 domains are important for CED-9(68–280) function. Substitution of the corresponding residues in the BH1 and BH2 domains of Bcl-2 abolished its death-protective effect and prevented it from forming a heterodimer with Bax, an interaction proposed to be important for protection against cell death by Bcl-2 (ref. 21).

A full-length CED-9(G169L, W214A) double mutant could still act as a substrate for CED-3 protease and gave partial protection against cell death (Fig. 1a, lanes 11 and 12; Table 2). Two further mutations (D44A, D67E) that disrupted both CED-3 cleavage sites abolished the remaining protective activity of CED-9(G169L, W214A) (Fig. 1a, lanes 13 and 14; Table 2). These results support the hypothesis that the two CED-3 cleavage sites in CED-9 confer protection against programmed cell death, just like the CED-3 cleavage site in the p35 protein⁹, indicating that CED-9 contains two distinct domains that can each inhibit cell death and which are both required for complete protection by CED-9.

We further tested this hypothesis by fusing the CED-9 N-terminal

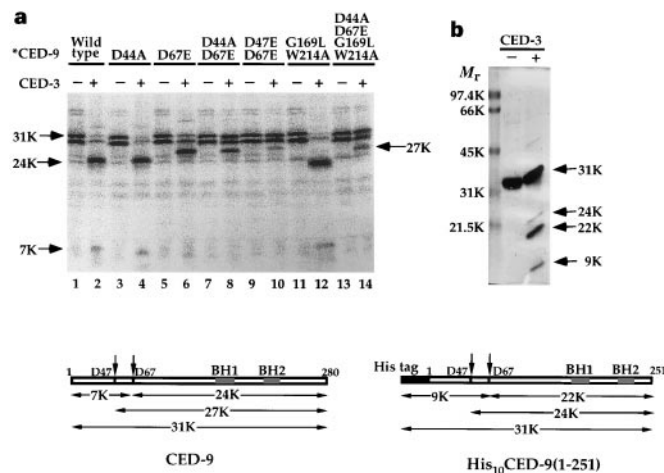


Figure 1 The *C. elegans* CED-9 protein is a substrate of the CED-3 protease. **a**, ³⁵S-methionine-labelled CED-9 protein (*CED-9) was incubated with (+) or without (-) 20 ng of purified CED-3 protease for 20 min at 30°C. The band seen below the full-length 31K CED-9 band may be an internal initiation product starting at methionine 20 of CED-9. **b**, Purified His₁₀CED-9(1–251) protein (10 µg) was incubated in CED-3 buffer without (-) or with (+) 160 ng purified CED-3 protease for 2 h at 30°C, resolved by 12.5% SDS-PAGE and stained with Coomassie brilliant blue. Diagrams underneath show the origins deduced for the detected protein bands: in **b**, the diagram is based on microsequencing analysis. Black box indicates the N-terminal tag, which includes ten histidines. Grey boxes (BH1 and BH2) indicate Bcl-2-homology domains²¹. Vertical arrows indicate proteolytic cleavage sites. Protein sizes are indicated. M_r , relative molecular masses of prestained protein markers (Bio-Rad).

Table 1 CED-3 cleavage sites are important for full CED-9 cell-death protective function

<i>hsp</i> construct*	Array*	No. of animals	Extra cells in anterior pharynx†	Range of extra cells‡
Vector	1	27	0.2 ± 0.4	0–1
	2	21	0.1 ± 0.3	0–1
CED-9	1	20	10.9 ± 1.3	8–13
	2	24	10.5 ± 1.8	7–14
	3	14	9.8 ± 3.2	4–14
CED-9(D44A)	1	27	7.6 ± 3.0	1–14
	2	21	8.2 ± 2.6	3–11
CED-9(D67E)	1	25	9.4 ± 2.8	3–14
	2	22	9.1 ± 2.8	3–14
	3	12	9.6 ± 1.9	7–13
CED-9(D44A, D67E)	1	23	2.5 ± 2.4	0–7
	2	35	4.1 ± 3.0	0–11
	3	23	4.0 ± 2.7	0–9
CED-9(D47E, D67E)	1	19	2.8 ± 2.6	0–8
	2	15	3.6 ± 2.4	0–8
CED-9(68–280)	1	17	4.2 ± 3.4	0–10
	2	23	4.5 ± 3.0	0–10
	3	21	4.9 ± 2.9	0–10
CED-9(1–67)	1	8	0.8 ± 1.2	0–3
	2	20	0.4 ± 0.6	0–2
	3	20	0.7 ± 1.0	0–3
CED-9(1–67) + CED-9(68–280)	1	14	4.2 ± 2.4	0–8
	2	14	4.4 ± 3.0	0–8
	3	10	3.3 ± 2.7	1–6

* The *hsp* constructs were co-injected at 100 µg ml⁻¹ each into wild-type N2 animals with pRF4 (at 50 µg ml⁻¹), which carries the dominant *rol-6* marker, as described⁸. Array indicates an extrachromosomal transgene carried by a given transgenic line.

† Heat-shock experiments were performed and scored as described⁸. Data shown are means ± s.e.m. All data depict results obtained after heat-shock treatment. *ced-3* mutants, presumably completely defective in cell death, have an average of 14 extra cells⁸.

region (amino acids 1 to 80), which includes the two CED-3 cleavage sites, to the N terminus of human Bcl-2. This CED-9(1–80)–Bcl-2 chimaeric protein resulted in significantly better protection against cell death than did Bcl-2 protein alone (Table 2). In addition, co-expression in *C. elegans* of baculovirus p35 protein (which contains a CED-3 cleavage site necessary for its cell-death protective function)⁹ and human Bcl-2 gave better protection than the separate proteins, indicating that p35, like CED-9(1–80) but unlike CED-9(68–280), can act additively with Bcl-2 (Table 2).

The *ced-9* gain-of-function mutation (G169E) enhances cell-death protection by *ced-9* (refs 1, 22). We found that CED-9(68–280) carrying the G169E mutation resulted in more effective protection against cell death than did CED-9(68–280) on its own, and was as effective as wild-type CED-9 (Table 2). This experiment indicates that the G169E mutation increases the potency of CED-9(68–280) in protecting against cell death.

We propose that CED-9 is a bifunctional cell-death inhibitor, protecting against programmed cell death in *C. elegans* by two distinct mechanisms. First, CED-9 inhibits cell death by interacting directly with the CED-3 death protease, possibly through a competitive mechanism like that proposed for baculovirus p35 protein⁹. Second, CED-9 also inhibits cell death through its C-terminal region by a mechanism similar to that used by mammalian Bcl-2. It has been proposed that CED-9 protects against CED-3 killing by acting at least in part via CED-4 (ref. 23), which like CED-3 is essential for programmed cell death in *C. elegans*^{24,25}. Furthermore, the CED-4 protein can interact with CED-3 and CED-9 *in vitro*^{26–28}. We have found that CED-9(68–280) is sufficient for interaction with CED-4 (D.X. and H.R.H., unpublished results). Our findings suggest that CED-9(68–280) inhibits programmed cell death in *C. elegans* as a result of a physical interaction with CED-4 and, furthermore, that Bcl-2 may inhibit cell death in mammals by means of a mammalian CED-4 homologue. □

Methods

Plasmid construction. We used standard methods of molecular biology²⁹ to generate various CED-9 constructs and their mutant derivatives.

Table 2 CED-9 may inhibit cell death through two different mechanisms

<i>hsp</i> construct*	Array*	No. of animals	Extra cells in anterior pharynx	Range of extra cells*
CED-9	1	20	10.9 ± 1.3	8–13
	2	24	10.5 ± 1.8	7–14
	3	14	9.8 ± 3.2	4–14
CED-9(68–280)	1	17	4.2 ± 3.4	0–10
	2	23	4.5 ± 3.0	0–10
	3	21	4.9 ± 2.9	0–10
Human Bcl-2	1	24	4.7 ± 2.8	1–12
	2	10	4.5 ± 1.8	1–7
	3	24	4.1 ± 2.3	0–8
CED-9(68–280) + hBcl-2	1	24	4.3 ± 2.6	1–9
	2	20	4.8 ± 2.7	0–9
	3	11	5.4 ± 1.8	2–8
CED-9(68–280; W214A)	1	16	2.4 ± 1.9	0–5
	2	18	2.3 ± 2.2	0–7
CED-9(68–280; G169L, W214A)	1	26	0.8 ± 1.2	0–5
	2	21	0.8 ± 1.0	0–4
	3	16	0.6 ± 1.0	0–4
CED-9(G169L, W214A)	1	20	2.4 ± 1.2	0–6
	2	20	2.2 ± 1.6	0–5
CED-9(D44A, D67E, G169L, W214A)	1	14	0.6 ± 0.8	0–2
	2	30	0.8 ± 0.9	0–3
CED-9(1–80)–hBcl-2	1	9	8.7 ± 2.7	4–12
	2	21	7.0 ± 2.7	3–12
	3	11	8.2 ± 2.6	3–12
p35†	1	17	8.9 ± 2.6	5–13
	2	16	8.6 ± 3.5	2–13
	3	21	8.8 ± 3.1	3–16
p35 + hBcl-2	1	13	11.2 ± 3.2	5–16
	2	21	12.8 ± 3.3	5–16
	3	30	12.1 ± 1.7	9–15
CED-9(68–280; G169E)	1	25	10.5 ± 3.3	3–16
	2	27	11.2 ± 2.4	6–15
	3	12	10.9 ± 1.9	7–13

* See descriptions in Table 1.

† Data from ref. 9.

Protease assays. Protease assays have been described¹⁸. Using purified His₁₀CED-9(1–251) protein and various CED-9 proteins synthesized in *E. coli* (N-terminal-tagged or C-terminal-tagged and purified, or untagged and unpurified), we were unable to demonstrate an inhibition of CED-3 protease activity (data not shown), perhaps because our *in vitro* conditions were unsuitable (such as the ionic conditions or protein concentrations, absent protein or non-protein cofactors, or inappropriate subcellular localization—other CED-9/Bcl-2 family members are associated with membranes³⁰).

Microsequencing analysis. Purification of recombinant His₁₀CED-9(1–251) protein from *E. coli* and microsequencing of its 22K and 24K CED-3 cleavage products were performed using methods previously described¹⁸.

Received 19 May; accepted 6 October 1997.

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Acknowledgements. We thank members of H.R.H.'s laboratory for comments about the manuscript and the MIT Biopolymers Laboratory for microsequencing analysis. D.X. was supported by postdoctoral fellowships from the Anna Fuller Fund and the Helen Hay Whitney Foundation and is a recipient of a Burroughs Wellcome Fund Career Award in the Biomedical Sciences. H.R.H. is an Investigator at the Howard Hughes Medical Institute.

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CRM1 is responsible for intracellular transport mediated by the nuclear export signal

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The discovery of nuclear export signals (NESs) in a number of proteins revealed the occurrence of signal-dependent transport of proteins from the nucleus to the cytoplasm^{1–14}. Although the consensus motif of the NESs has been shown to be a leucine-rich, short amino-acid sequence^{2,6,7}, its receptor has not been identified. A cytotoxin leptomycin B (LMB) has recently been suggested to inhibit the NES-mediated transport of Rev protein¹⁵. Here we show that LMB is a potent and specific inhibitor of the NES-dependent nuclear export of proteins. Moreover, we have found a protein of relative molecular mass 110K (p110) in *Xenopus* oocyte extracts that binds to the intact NES but not to the mutated, non-functional NES. The binding of p110 to NES is inhibited by LMB. We show that p110 is CRM1, which is an evolutionarily conserved protein^{16–18} originally found as an essential nuclear protein in fission yeast¹⁶ and known as a likely target of LMB¹⁹. We also show that nuclear export of a fission yeast protein, Dsk1, which has a leucine-rich NES, is disrupted in wild-type yeast treated with LMB or in the *crm1* mutant. These results

indicate that CRM1 is an essential mediator of the NES-dependent nuclear export of proteins in eukaryotic cells.

A peptide corresponding to the NES sequence of mitogen-activated protein kinase kinase (MAPKK)⁵ was crosslinked to ovalbumin (OVA), and the resultant conjugate (KK–NES–OVA) was injected with rhodamine-labelled bovine serum albumin (RITC–BSA) into the nuclei of fibroblastic cells. Ten minutes after the injection, almost all the KK–NES–OVA was excluded from the nucleus, whereas RITC–BSA remained in the nucleus⁵ (Fig. 1a). When cells were pretreated with 0.2 ng ml^{–1} LMB, both KK–NES–OVA and RITC–BSA were present exclusively in the nucleus after the injection (Fig. 1). In more than 95% of the injected cells, the nuclear export of KK–NES–OVA was inhibited completely by 0.2 ng ml^{–1} LMB. This strong inhibition of the nuclear export was observed at concentrations of 0.2–20 ng ml^{–1} LMB (data not shown). A conjugate between OVA and the NES peptide of Rev protein (Rev–NES–OVA) moved to the cytoplasm within 10 min when injected into the nucleus, and this nuclear export was also strongly inhibited by LMB (Fig. 1b). In contrast, the nuclear import of nuclear localization signal (NLS)-conjugated BSA (NLS–BSA) was not inhibited by LMB, even at a concentration of 20 ng ml^{–1} (Fig. 1c). The NES of MAPKK was found to compete with the NES of PKI-α⁵ and the NES of Rev (M.F., S.A. & E.N., unpublished data). The above data, together with the previous observation that LMB disrupted the NES-directed cytoplasmic localization of Rev¹⁵, therefore indicate that LMB specifically inhibits the nuclear export of proteins, which is mediated by the NES rich in hydrophobic residues (usually leucine). We therefore examined the effect of LMB on the subcellular distribution of MAPKK, the cytoplasmic localization of which is determined by its NES⁵. Within 5 min of LMB treatment, MAPKK began to appear in the nucleus, and by 20–30 min MAPKK was distributed evenly throughout the cell (Fig. 1d). In about 30% of the cells, MAPKK appeared to be accumulated in the nucleus after 60 min. This pattern of intracellular distribution of MAPKK is similar to that of the NES-disrupted MAPKK in cells without LMB⁵. Thus LMB is shown to inhibit the NES-dependent nuclear export of proteins.

To determine which protein(s) binds specifically to the NES, we searched for a protein that binds to the intact NES sequence of MAPKK, but not to the non-functional, mutated (leucine to alanine) NES sequence. The fusion proteins between glutathione S-transferase (GST) and the amino-terminal region of MAPKK (residues 1–60) that had either an intact wild-type NES (WT–NES, residues 33–44) or a leucine-to-alanine mutated NES (LA–NES) were immobilized on glutathione Sepharose and incubated with extracts obtained from *Xenopus* oocytes. After washing, the bound proteins were eluted with glutathione. SDS–polyacrylamide gel electrophoresis (SDS–PAGE) analysis of eluted fractions showed that a protein with an apparent relative molecular mass of 110,000 (*M_r* 110K), p110, binds to the WT–NES, but not to LA–NES (Fig. 2a). The binding of p110 to WT–NES beads was inhibited by free NES peptide (corresponding to residues 27–44 of MAPKK), but not by free, mutated NES (LA–NES) peptide (Fig. 2c, left). Furthermore, the binding was completely blocked in the presence of LMB (Fig. 2d, left). These results suggest that p110 is a candidate for a transport factor for NES-bearing proteins.

A fission yeast *Schizosaccharomyces pombe* gene, *crm1*⁺, which encodes a nuclear protein of *M_r* 115K, was originally isolated by complementation of a cold-sensitive mutant causing deformed chromosome structure¹⁶, and has been suggested to be a target of LMB¹⁹. A human homologue of CRM1 has recently been identified¹⁸ and shown to bind to the oncogenic nucleoporin CAN/Nup214 (ref. 20) and to move between the nuclear pore and the nucleoplasm. It has therefore been proposed that CRM1 may be a soluble nuclear transport factor¹⁸. Human CRM1 was reported to have an *M_r* of 112K on SDS–PAGE^{18,20}, very similar to p110. We therefore hypothesized that p110 might be *Xenopus* CRM1. To test this

hypothesis, we used a specific antibody against *S. pombe* Crm1 (ref. 16) (Fig. 2b). This antibody had previously been shown to cross-react with a putative mammalian CRM1 protein¹⁶. In immunoblotting analysis, the antibody reacted specifically with a 110K protein in the eluate from the intact NES beads (Fig. 2b, WT-NES) that was the same size as the p110 identified by silver staining (Fig. 2a). The antibody-recognizable 110K protein did not bind to the non-functional NES (Fig. 2b, LA-NES). The binding of this protein to the intact NES beads was inhibited by free NES peptide, but not by free LA-NES peptide (Fig. 2c, right), and was completely blocked by LMB (Fig. 2d, right). Four internal peptides from p110 were sequenced and found to be identical to the sequence of human CRM1 (ref. 18; S.A., A. Iwamatsu, M.F. & E.N., unpublished data). Furthermore, purified recombinant human CRM1 bound to WT-NES, but not to LA-NES (Fig. 2e). These results indicate that p110 is CRM1, and that it binds specifically to NES in an LMB-sensitive

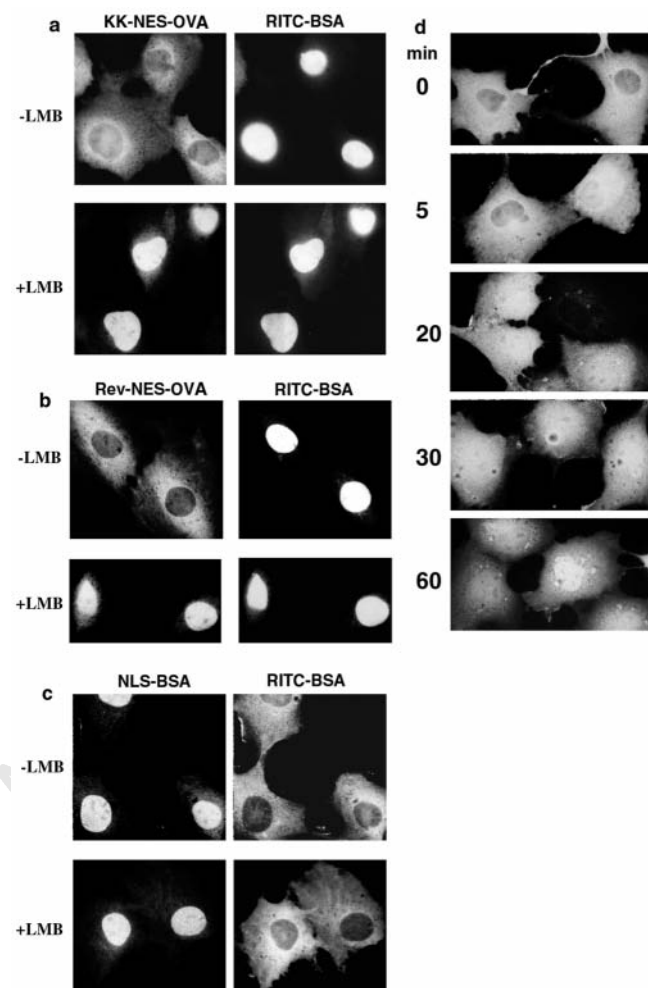


Figure 1 LMB is an inhibitor of the NES-dependent nuclear export of proteins. **a, b**, KK-NES (residues 32–44 of MAPKK)–OVA (2.4 mg ml⁻¹) or Rev-NES (residues 73–84 of Rev)–OVA (2.4 mg ml⁻¹) was injected with RITC-labelled bovine serum albumin (RITC-BSA, 1.0 mg ml⁻¹) into the nucleus of rat 3Y1 cells. The cells were incubated in the presence (+) or absence (–) of LMB (0.2 ng ml⁻¹). LMB was added to the medium 30 min before injection. The cells were fixed 10 min after injection and stained with an anti-OVA antibody⁵. **c**, A conjugate between the NLS of SV40 T antigen and FITC-labelled BSA (NLS-BSA, 1.0 mg ml⁻¹)²⁶ was injected with RITC-BSA (1.0 mg ml⁻¹) into the cytoplasm of rat 3Y1 cells. The cells were incubated at 37°C in the presence or absence of LMB (20 ng ml⁻¹). The cells were fixed 30 min after injection and observed. **d**, COS-7 cells were transiently transfected with a plasmid containing haemagglutinin (HA)-tagged *Xenopus* wild-type MAPKK. At 16 h after transfection, cells were treated with LMB (0.2 ng ml⁻¹). Cells were then fixed at the indicated time and stained with an anti-HA monoclonal antibody (12CA5).

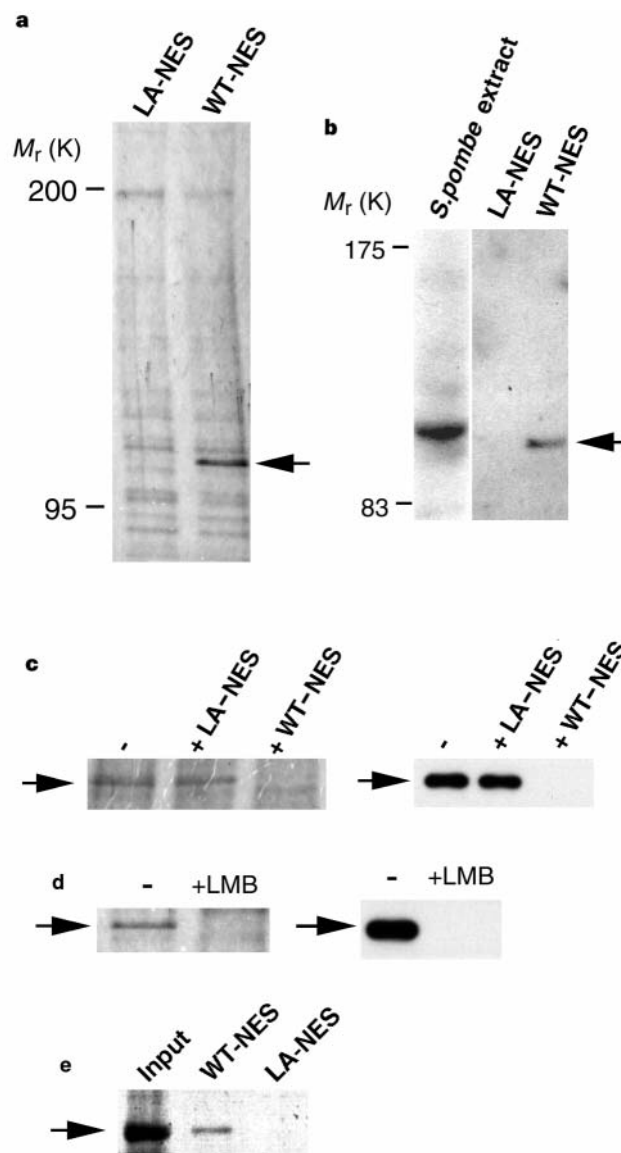


Figure 2 The p110 protein binds specifically to NES in an LMB-sensitive manner. **a**, *Xenopus* oocyte extracts were loaded onto a column containing glutathione-Sepharose beads to which a GST fusion protein with an intact, wild-type NES (WT-NES) of MAPKK or a non-functional, mutated NES (LA-NES) was attached. After washing, bound proteins were eluted with glutathione. The eluate was subjected to SDS-PAGE followed by silver staining. A 110K protein (indicated by an arrow) bound specifically to the WT-NES beads. **b**, Immunoblotting analysis of the eluted fractions. The eluate from the glutathione-Sepharose column containing GST-WT-NES or GST-LA-NES was subjected to immunoblotting analysis using an antibody specific to *S. pombe* Crm1. An arrow indicates the position of p110 that was detected by silver staining. As a control, *S. pombe* extracts were used, where a single band with an apparent M_r of 115K was detected. **c**, The effect of free NES peptides on the interaction of p110 with the NES column. *Xenopus* oocyte extracts were mixed without (–) or with (+) 2 mg ml⁻¹ of a wild-type NES peptide (WT-NES peptide) corresponding to residues 27–44 of MAPKK or a mutated, non-functional NES peptide (LA-NES peptide), and the mixture was then loaded onto the WT-NES column as in **a**. The bound proteins were analysed by SDS-PAGE followed by silver staining (left) or by immunoblotting with anti-Crm1 antibody (right). **d**, *Xenopus* oocyte extracts were incubated in the presence or absence of LMB (1 µg ml⁻¹) at 4°C and were then loaded onto the WT-NES column. After washing, the bound proteins were eluted and analysed by SDS-PAGE followed by silver staining (left) or by immunoblotting with anti-Crm1 antibody (right). Arrows indicate the position of p110 that was detected by silver staining in **a, e**. Binding of purified CRM1 to WT-NES, but not to LA-NES. Purified recombinant human CRM1 (Input) was mixed with either GST-WT-NES (WT-NES) or GST-LA-NES (LA-NES), precipitated with glutathione Sepharose, and analysed by SDS-PAGE followed by protein staining. An arrow indicates CRM1.

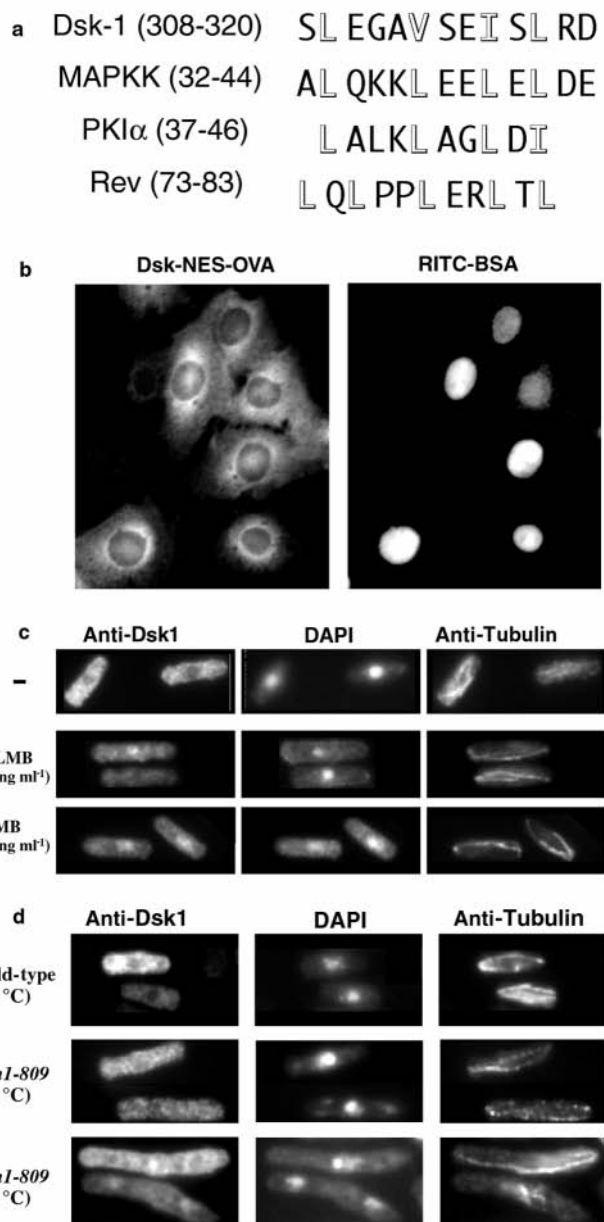


Figure 3 Disruption of cytoplasmic localization and nuclear exclusion of Dsk1 in a fission-yeast *crm1* mutant. **a**, Comparison of the NES sequences of Dsk1, MAPKK, PKI and Rev. Hydrophobic residues that seem to be important for NES activity are written in outline letters. **b**, A sequence (residues 308–320) of Dsk1 can function as an NES. Dsk1–NES (residues 308–320)–OVA was injected with RITC–BSA into the nuclei of rat 3Y1 cells. At 30 min after injection, cells were fixed and stained with anti-OVA antibody. **c**, Effect of LMB on the subcellular localization of Dsk1 in fission-yeast cells. wild-type cells carrying a plasmid with the *dsk1*⁺ gene were incubated in the presence or absence of LMB (5 or 50 ng ml⁻¹) at 33 °C for 3 h. Cells were fixed and stained with anti-Dsk1 antibody, anti-tubulin monoclonal antibody (TAT1) or DAPI. The percentage of cells showing nuclear presence of Dsk1 was 5.3% (– LMB), 68% (5 ng ml⁻¹ LMB) and 89% (50 ng ml⁻¹ LMB). **d**, Subcellular localization of Dsk1 in a *crm1* cold-sensitive mutant. Wild-type cells or *crm1* mutant cells (*crm1-809*)¹⁶ carrying the *dsk1*⁺ gene plasmid were grown to early log phase at 33 °C, and then shifted to 20 °C and cultured for 6 h. These cells were fixed and stained as described in **c**. The percentage of cells showing Dsk1 in the nucleus was 4.2% (WT, 20 °C), 70% (*crm1-809*, 33 °C) and 92% (*crm1-809*, 20 °C). Previous results²² demonstrated that Dsk1 is present exclusively in the cytoplasm during interphase and is accumulated in the nucleus during the mitotic phase. Anti-tubulin staining showed that the cells shown here contained cytoplasmic microtubules, characteristic of interphase cells of *S. pombe*, indicating that the nuclear accumulation of Dsk1 observed in **c** and **d** was not due to entry into the mitotic phase.

manner, and thus they raise the possibility that CRM1 functions as a mediator for the NES-dependent transport of proteins.

To test this possibility, we examined nuclear export in the fission yeast *S. pombe*. Previous work demonstrated that an *S. pombe* protein kinase, Dsk1, the mammalian homologue of which is SRPK1, a kinase acting on splicing factors²¹, localizes exclusively to the cytoplasm during interphase. In contrast, a mutant Dsk1 lacking a spacer region (residues 228–352) localizes to both the nucleus and the cytoplasm²². We found a short amino-acid sequence (Fig. 3a) in the spacer region that coincided with the consensus motif of the hydrophobic residue-rich NES. We then synthesized a peptide corresponding to this sequence, conjugated it to OVA, and injected the resultant conjugate (Dsk1–NES–OVA) into the nucleus of rat 3Y1 cells. Dsk1–NES–OVA was completely excluded from the nucleus (Fig. 3b), indicating that this sequence in the spacer region of Dsk1 is an NES that might determine cytoplasmic localization of Dsk1 in *S. pombe*. We then examined the effect of LMB on the subcellular distribution of Dsk1 in *S. pombe*. The staining with an anti-Dsk1 antibody showed that, in the absence of LMB, the expressed Dsk1 was present exclusively in the cytoplasm during interphase, as reported previously²², whereas it became localized to both the nucleus and the cytoplasm in the presence of LMB (Fig. 3c). These results suggest that the hydrophobic residue-rich NES functions in *S. pombe*, and that LMB also acts as an inhibitor of the NES-dependent transport in *S. pombe*.

To test directly whether *S. pombe* CRM1 protein is involved in the process of the NES-dependent nuclear export of proteins, we determined the subcellular distribution of Dsk1 in the *crm1* cold-sensitive mutant (*crm1-809*)¹⁶. The *dsk1*⁺ gene was introduced into the *crm1-809* cells, and the cells producing Dsk1 protein were incubated at the permissive or restrictive temperature. Immunostaining with anti-Dsk1 antibody showed that Dsk1 appeared in the nucleus even at the permissive temperature (Fig. 3d). At the restrictive temperature, Dsk1 was present diffusely throughout the cell, including the nucleus, and the nuclear accumulation of Dsk1 was observed in some cells (Fig. 3d). These results suggest that CRM1 is required for the NES-dependent nuclear export of Dsk1. It is not unexpected that the cytoplasmic localization of Dsk1 is disrupted even at the permissive temperature in the *crm1-809* cells, as several mutant phenotypes were previously observed in this mutant at the permissive temperature^{16,17}. CRM1 may function primarily as a mediator of NES-dependent nuclear export of proteins in *S. pombe*, and the defect in CRM1 might give rise to a variety of phenotypes as secondary effects, although it is also possible that CRM1 has several functions.

Our genetic and biochemical analyses strongly suggest that CRM1, a ubiquitous nuclear protein that is highly conserved during evolution^{16–18} and is essential in fission yeast^{16,17}, is a transport factor, or a component of the transporter, for the NES-dependent nuclear export of proteins in eukaryotic cells from yeast to vertebrates. The previously reported candidates for Rev NES receptors, the yeast Rip protein²³ and the related human RIP/RAB protein^{24,25}, might interact with NES through CRM1, and may cooperate with CRM1 in nuclear export. The identification of an essential mediator for nuclear export in this study will facilitate further analyses of the molecular mechanisms of nuclear export. *Note added in proof*: While this paper was under review, three papers appeared indicating that CRM1 is a nuclear export receptor^{27–29}. □

Methods

Microinjection and cell staining. Cultures of 3Y1 cells, microinjection and conjugation of synthetic peptides to OVA were performed as described⁵. After injection, cells were fixed and stained as described⁵.

Preparation of oocyte extracts. *Xenopus* oocytes were dissected manually and treated with collagenase (2 mg ml⁻¹) in Ca²⁺-free modified Barth solution containing (in mM) 88 NaCl, 1 KCl, 2.5 NaHCO₃, 10 HEPES, pH 7.4, 0.82

MgSO₄, 0.33 Ca(NO₃)₂, 0.4 CaCl₂. Defolliculated oocytes were washed with A-buffer containing (in mM) 20 HEPES, pH 7.3, 110 CH₃COOK, 2 Mg(CH₃COO)₂, 0.5 EGTA, 2 dithiothreitol, 1 PMSF and 20 µg ml⁻¹ aprotinin. The oocytes were disrupted in 3 volumes of A-buffer with a Polytron homogenizer. The homogenate was then centrifuged for 15 min at 27,000g. The supernatant was further centrifuged for 1 h at 125,000g. The resultant supernatant was used as *Xenopus* oocyte extracts.

Affinity chromatography. The fusion proteins between GST and the N-terminal region (residues 1–60) of MAPKK with either an intact wild-type NES (WT–NES) or a non-functional, disrupted NES (LA–NES) were prepared as described²⁶. *Xenopus* oocyte extracts were loaded onto a 0.2 ml glutathione-Sepharose column containing 5 mg of each of the GST fusion proteins. The column was washed with 0.6 ml of A-buffer, and bound proteins were eluted by the addition of 200 µl of A-buffer containing 10 mM glutathione. Recombinant human CRM1 was produced in bacteria and purified (M.Y. *et al.*, manuscript in preparation). The purified human CRM1 was mixed with either GST–WT–NES or GST–LA–NES in A-buffer containing 70 mM NaCl at 4°C, and the mixture was subjected to precipitation with glutathione Sepharose. After three washes, bound proteins were eluted with 10 mM glutathione and analysed by SDS–PAGE followed by Coomassie staining.

Immunoblotting. The eluates from the above column were subjected to SDS–PAGE and transferred to polyvinylidene difluoride (PVDF) membrane. After blocking with 3% BSA, membranes were incubated with anti-S. *pombe* CRM1 antibody¹⁶ at a dilution of 1 : 500 at 4°C for 14 h. Reacted proteins were detected by HRP–anti-rabbit IgG antibody.

S. pombe indirect immunofluorescence. Wild-type yeast cells (972^h) carrying a plasmid with the *dskl*⁺ gene²² were grown to early log phase, and further incubated in the presence or absence of LMB at the indicated concentrations for 3 h. Then *crm1*–809 cells were transformed as described²² with a multicopy plasmid (pDS113–6) that contains the *dskl*⁺ gene. Exponentially growing cells carrying a plasmid with the *dskl*⁺ gene at 33°C were transferred to 20°C for 6 h. The cells were then fixed and processed for indirect immunofluorescence as described²². Cells overexpressing Dsk1 were used in these experiments, because endogenous Dsk1 is not expressed at levels high enough to be detected by our antibody.

Received 14 July; accepted 13 October 1997.

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Acknowledgements. We thank K. Kumada for comments and discussion, and M. Watanabe for preparation of Dsk1–NES-OVA. This work was supported by grants from the Ministry of Education, Science and Culture of Japan (E.N.) and the Japan Science Technology Corporation (M. Yanagida).

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DNA binding and transcriptional repression by DAX-1 blocks steroidogenesis

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Mutations in the *DAX-1* gene are responsible for congenital X-linked adrenal hypoplasia, a disease that is associated with hypogonadotropic hypogonadism^{1,2}. *DAX-1* expression is tissue-specific and is finely regulated throughout development^{3–5}, suggesting that it has a role in both adrenal and gonadal function. *DAX-1* is an unusual member of the nuclear-receptor superfamily of transcription factors which contains no canonical zinc-finger or any other known DNA-binding motif¹. Binding sites for *DAX-1* are found in the promoters of the *dax-1* and *StAR* (for steroidogenic acute regulatory protein) genes. Here we show that *DAX-1* binds DNA and acts as a powerful transcriptional repressor of *StAR* gene expression, leading to a drastic decrease in steroid production. We provide *in vitro* and *in vivo* evidence that *DAX-1* binds to DNA hairpin structures. Our results establish *DAX-1* as the first member of the nuclear receptor superfamily with novel DNA-binding features and reveal that it has regulatory properties critical to the understanding of its physiological functions.

DAX-1 has an amino-terminal domain that is distinct from all other nuclear receptors (Fig. 1a). We have found that *DAX-1* efficiently recognizes DNA hairpin structures *in vitro* and that binding is equally efficient to stems composed of 10 to 24 nucleotides, but is less efficient with shorter stems (Fig. 1b). The sequence of the loop influences *DAX-1* binding: an adenine-rich sequence shows reduced binding compared with loops rich in cytosines or thymines (Fig. 1c), although the presence of the loop itself is crucial (lane 6 in Fig. 1b).

Methylation interference indicates that *DAX-1* does not predominantly interact with the major groove of the DNA helix.

Instead, DAX-1 binding is greatly diminished by increasing concentrations of distamycin A (Fig. 1d) or chromomycin A3, indicative of interaction with the minor groove. These DNA-binding properties are reminiscent of HMG-box proteins⁶⁻⁹. DAX-1 binding, however, requires the presence of a single-stranded loop, suggesting the presence of a motif necessary for this function in its N terminus.

We searched for possible hairpins in gene promoters likely to be under DAX-1 regulation. Two apparently stable hairpins are present within the *dax-1* gene promoter, one (H1) between positions -121 and -75 (stem of 13 nucleotides, loop of 21 nucleotides) and another (H2) between -127 and -98 (stem and loop of 10 nucleotides). Both hairpins bind DAX-1 (Fig. 2). H1-loop mutants (MutLH1), which still allow hairpin formation, also bind DAX-1.

The spatial and temporal pattern of DAX-1 expression in the adrenal cortex and Leydig cells suggests that it has a role in steroidogenesis, a process regulated by StAR¹⁰⁻¹². Another DAX-1-binding site is present in the *StAR* gene promoter (positions -61/-27) (Fig. 3). DAX-1 specifically binds to the *StAR* hairpin. Binding is inhibited by an anti-DAX-1 antibody and distamycin A.

To determine whether the predicted hairpins are formed, two stem mutants were generated, MutH1 and MutH2 (Fig. 2). Linear DNA polymerase extension of individual strands revealed stops at position -121 on one strand (Fig. 4a) and at -75 on the other (Fig. 4b), corresponding to the boundaries of *dax-1* H1. These stops were

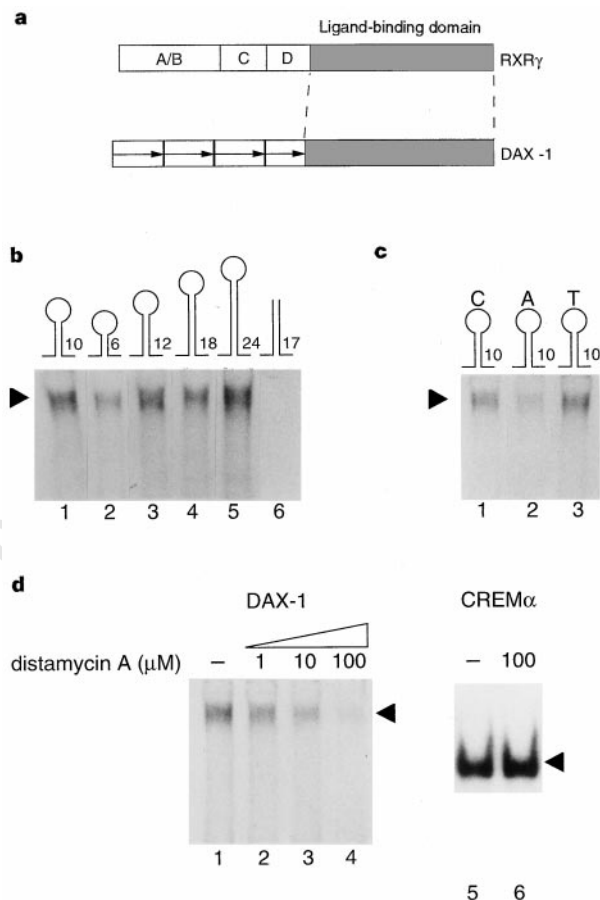


Figure 1 Preferential binding of DAX-1 to hairpin structures. **a**, General structure of DAX-1 and comparison with the retinoid-X receptor RXRγ. N-terminal amino acid repeats¹ are indicated by horizontal arrows. **b**, Binding analysis using hairpins with different stem lengths. Binding is weaker with a short stem (lane 2) and absent if the loop is missing (lane 6). **c**, Effect of the nucleotide composition of the loop upon DAX-1 DNA binding. Binding is weaker when loops are rich in adenine (lane 2). **d**, Interaction of DAX-1 with the minor groove of the DNA. Binding of DAX-1 (lane 1) is inhibited by increasing concentrations of distamycin A. No effect is seen on CREMα binding to a cAMP-response element (CRE; lanes 5, 6).

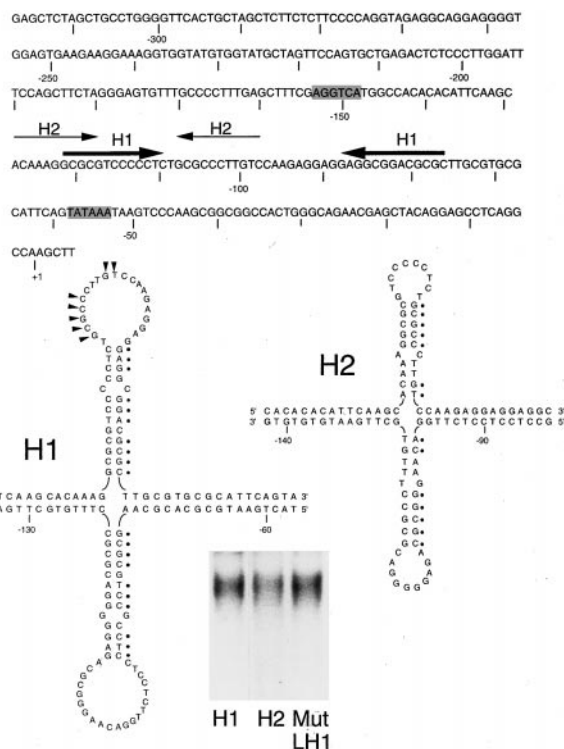


Figure 2 Presence of inverted repeats in the mouse *dax-1* promoter. Inverted repeats are indicated. H1 hairpin is defined by inverted repeats between nucleotides -75 to -87 and -109 to -121. The H2 hairpin is defined by inverted repeats between positions -99 to -108 and -119 to -128. The DAX-1 start codon is defined as +1. The location of the SF-1 binding site (AGGTCA) at position -150 is indicated. Dots on the hairpins represent substitutions generating the mutated MutH1 and MutH2 promoters. Arrowheads in the loop of H1 represent nucleotide substitutions generating MutLH1. All three hairpins bind DAX-1.

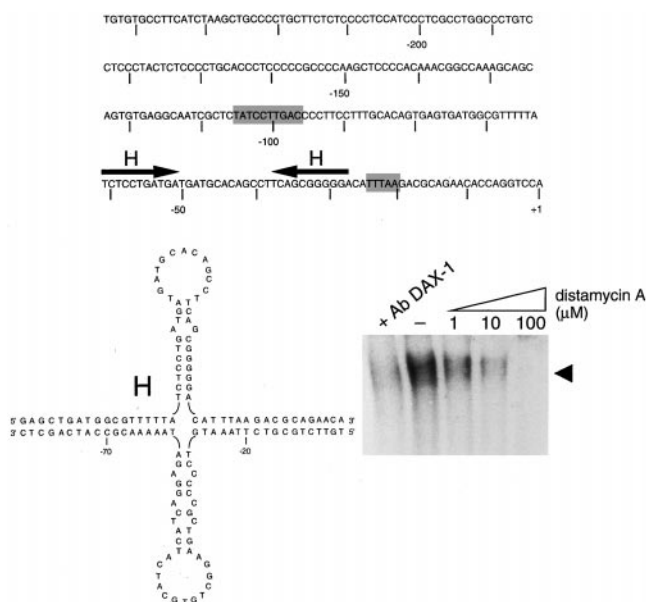


Figure 3 Presence of hairpin H in the *StAR* promoter. Inverted repeats are indicated and form hairpin H (nucleotides -61 and -27). The initiation site is defined as +1. An SF-1 binding site and the TATA-like sequence are indicated. This hairpin binds DAX-1. Formation of the complex is greatly reduced by an anti-DAX-1 antibody as well as by addition of distamycin A.

not detected in the MutH1 promoter (Fig. 4a, b). As no other major stops exist, we conclude that H1 formation is favoured over H2. KMnO_4 , which reacts with unstacked or unpaired bases¹³, modifies adenine -108 in the loop of H1, but not in that of the MutH1 promoter (Fig. 4c). The hairpin appears to exist *in vivo*, as confirmed by treatment of transfected cells with chloroacetaldehyde (CAA). Nucleotides at -105 and -106 were exposed and modified by CAA in H1 but not in the MutH1 promoter (Fig. 4d).

The transcriptional effect of DAX-1 was evaluated by transient expression in transfected cells. At position -150 of the mouse *dax-1* promoter there is an SF-1 binding site. SF-1 activates the *dax-1* promoter, as well as the DelH1 mutant lacking H1 (Fig. 5a). DAX-1

moderately represses the basal activity of both wild-type and DelH1 promoters, whereas it drastically blocks SF-1-mediated activation. Note that SF-1 activation of DelH1 is only marginally affected by DAX-1 (Fig. 5a, c).

Transcription of the human *StAR* gene is inducible by cyclic AMP¹⁴. We transfected steroidogenic Y-1 cells with a *StAR* promoter¹⁴ reporter construct and observed inducibility by forskolin (Fig. 5b). DAX-1 repressed both basal- and cAMP-induced promoter activity. A mutated *StAR* promoter (DelH) with the H hairpin deleted is still cAMP-inducible, but is not repressed by DAX-1 (Fig. 5b, d).

DAX-1 also exerts its repression function in a heterologous

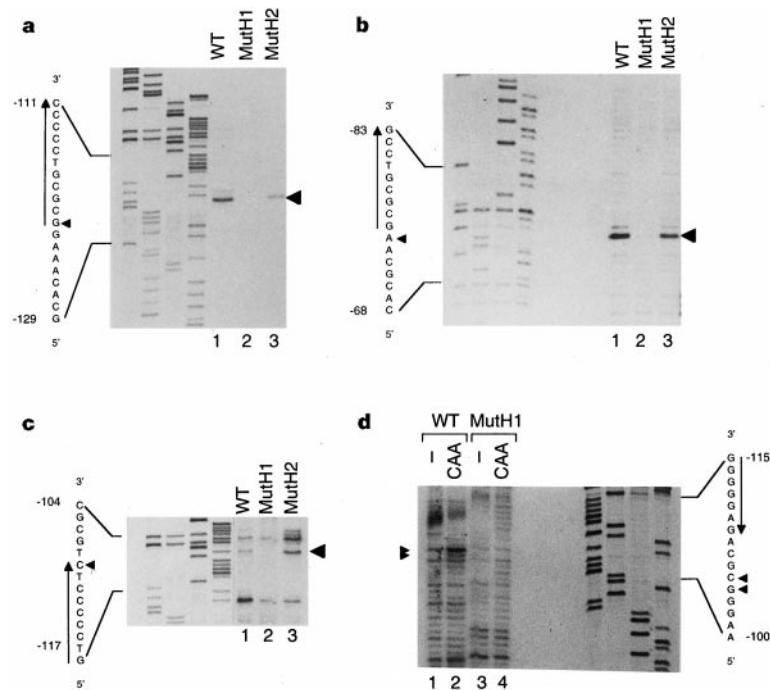


Figure 4 Formation of H1 in the mouse *dax-1* promoter. **a**, Extension of the sense strand of the wild-type and MutH2 promoters generates a polymerase stop at position -121 (lanes 1 and 3). There is no stop in MutH1 (lane 2). **b**, Extension of the antisense strand shows a polymerase stop at position -75 (lane 1). This stop is observed using MutH2 (lane 3) but not MutH1 (lane 2). **c**, Presence of unpaired nucleotides between the H1 inverted repeats. Plasmids carrying promoter sequences were treated with KMnO_4 . Extension of antisense sequences shows a modified adenine at -108 (lane 1) in the wild-type promoter. The same stop is in MutH2 (lane 3) but not in MutH1 (lane 2). **d**, *In vivo* treatment of plasmids carrying wild-type and MutH1 promoters with CAA reveals unpaired bases (-104 and -105) exposed to the reagent (lane 2). These stops are absent (-104) or reduced (-105) when the cells are not treated with CAA (lane 1) and absent in the MutH1 promoter (lanes 3, 4). Vertical arrows along the sequences depict the boundaries of the hairpin.

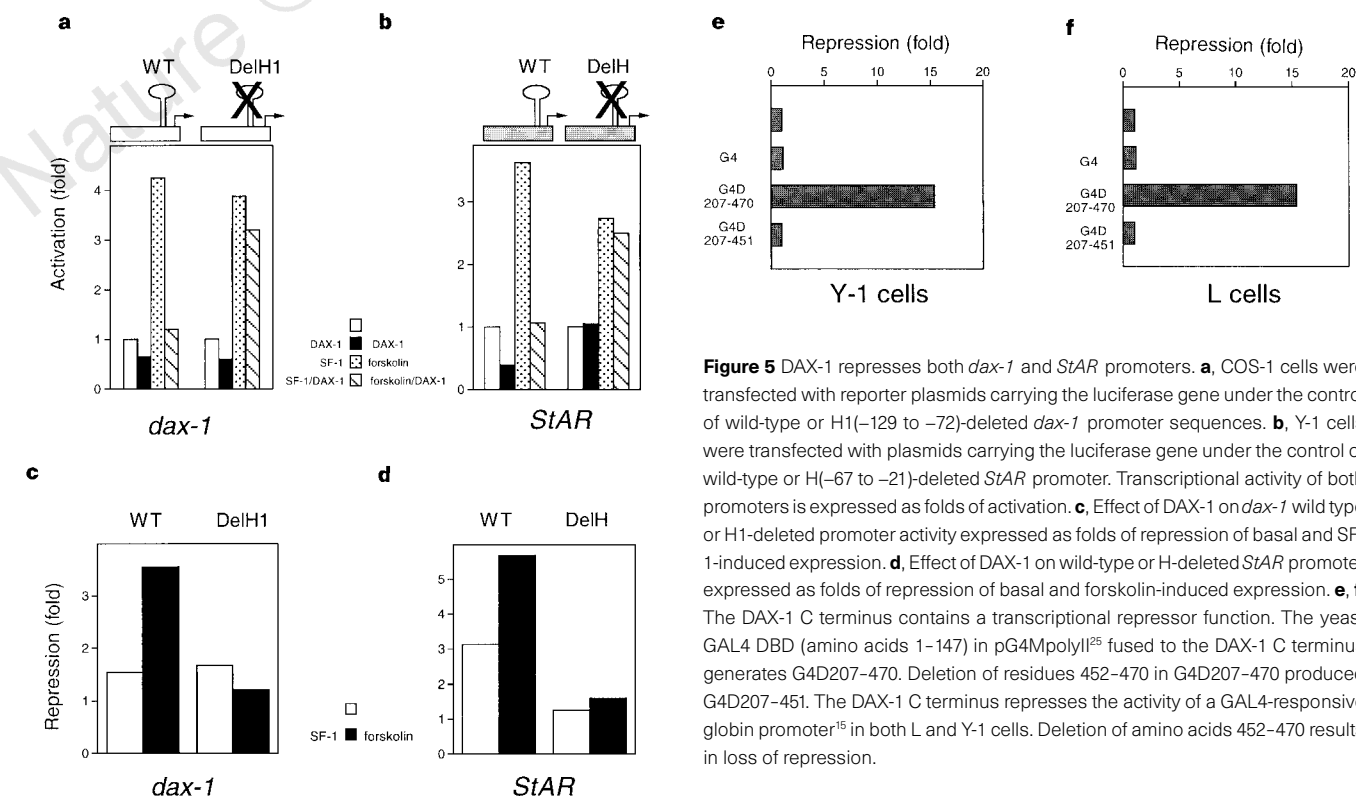


Figure 5 DAX-1 represses both *dax-1* and *StAR* promoters. **a**, COS-1 cells were transfected with reporter plasmids carrying the luciferase gene under the control of wild-type or H1(-129 to -72)-deleted *dax-1* promoter sequences. **b**, Y-1 cells were transfected with plasmids carrying the luciferase gene under the control of wild-type or H(-67 to -21)-deleted *StAR* promoter. Transcriptional activity of both promoters is expressed as folds of activation. **c**, Effect of DAX-1 on *dax-1* wild type or H1-deleted promoter activity expressed as folds of repression of basal and SF-1-induced expression. **d**, Effect of DAX-1 on wild-type or H-deleted *StAR* promoter expressed as folds of repression of basal and forskolin-induced expression. **e**, **f**, The DAX-1 C terminus contains a transcriptional repressor function. The yeast GAL4 DBD (amino acids 1-147) in pG4MpolyI²⁵ fused to the DAX-1 C terminus generates G4D207-470. Deletion of residues 452-470 in G4D207-470 produced G4D207-451. The DAX-1 C terminus represses the activity of a GAL4-responsive globin promoter¹⁵ in both L and Y-1 cells. Deletion of amino acids 452-470 results in loss of repression.

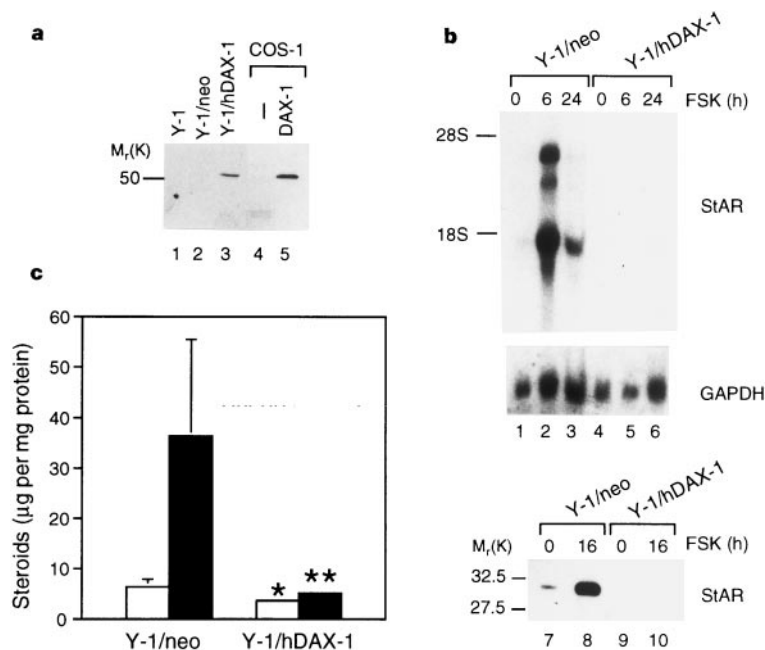


Figure 6 Expression of DAX-1 in adrenal Y-1 cells blocks steroidogenesis. **a**, Western blot showing DAX-1 expression in Y-1 (lane 1), Y-1/neo (lane 2) and Y-1/hDAX-1 cells (lane 3). Lane 4: mock-transfected cells; lane 5: cells transfected with pSG.DAX-1. **b**, *StAR* RNA expression (lanes 1–6) in Y-1/neo (lanes 1–3) and Y-1/hDAX-1 (lanes 4–6) cells in basal conditions, 6 and 24 h after forskolin stimulation. GAPDH expression is shown as control. *StAR* protein expression in Y-1/neo (lanes 7, 8) and Y-1/hDAX-1 (lanes 9, 10) cells both in basal conditions (lanes 7, 9) and after 16 h of forskolin stimulation (lanes 8, 10). **c**, Steroid production in Y-1/neo and Y-1/hDAX-1 cells. White bars indicate basal steroid production; black bars show levels of released steroids after 16 h of forskolin induction. The histogram shows the mean and the s.e.m. of six duplicated experiments. A statistically significant difference (Wilcoxon rank-sum test) between Y-1/neo and Y-1/hDAX-1 cells is indicated with one asterisk ($P = 0.046$) for basal steroid production and with two asterisks ($P = 0.025$) for forskolin-stimulated steroid production.

system. Fusion of the DAX-1 carboxy terminus to the GAL4 DNA-binding domain (DBD) results in a protein that efficiently represses a globin promoter containing GAL4-binding sites. Deletion of DAX-1 amino acids 452–470 results in decreased repression (Fig. 5e, f)¹⁵.

Y-1 adrenocortical tumour cells constitute a model to study the biochemical mechanisms of steroid hormone production¹⁶. Y-1 cells do not endogenously express DAX-1 (Fig. 6a), so we produced stably transfected clones expressing DAX-1 (Y-1/hDAX-1) and control cells expressing only the neomycin-resistance gene (Y-1/neo).

StAR basal expression is very low in Y-1/neo cells, but is rapidly and transiently induced upon forskolin treatment. *StAR* transcripts and protein are undetectable in Y-1/hDAX-1 cells after forskolin stimulation (Fig. 6b). Given the direct link between *StAR* function and steroidogenesis^{10–12,17}, we tested steroid production in Y-1/hDAX-1 cells and found a 5–6-fold increase in steroid production in Y-1/neo cells after forskolin stimulation. Both basal and cAMP-stimulated steroidogenesis are blocked in Y-1/hDAX-1 cells (Fig. 6c). Forskolin-stimulated steroid production is affected to a greater degree than basal steroidogenesis. cAMP signalling remains functional in the Y-1/hDAX-1 clones. SF-1, which is implicated in *StAR* transcriptional regulation^{18,19}, is equally expressed in Y-1/hDAX-1 and Y-1/neo cells (not shown).

We have shown that DAX-1 binds *in vitro* to hairpin structures which are also formed *in vivo* in transiently transfected cells. This binding shows no strong sequence specificity. These unusual DNA-binding properties are reminiscent of HMG-box proteins, such as SRY, a transcription factor whose function is linked to sex determination in humans and mice²⁰. However, whereas HMG proteins can bind to four-way DNA junctions, DAX-1 is unable to if the loop is missing (Fig. 1b). Moreover, predictions of the secondary structure of the N-terminal domain show a majority of β -sheets with very few regions in an α -helical conformation.

Our results show that DAX-1 represses *StAR* expression and consequently suppresses steroidogenesis by binding to a hairpin in the *StAR* promoter. DAX-1 is expressed in gonadal steroidogenic cells and may influence sex determination by controlling the production of sexual steroid hormones in the embryo. The DAX-1 gene is positioned in the X-chromosome region associated with double-dosage sex reversal²¹.

The proximity of SF-1- and DAX-1-binding sites in the *dax-1* and

StAR promoters suggests a mechanism of allosteric inhibition by which DAX-1 might impair SF-1 binding. A direct *in vitro* interaction of DAX-1 and SF-1 in the absence of DNA has recently been described²² which we have confirmed (unpublished results, and K. Parker, personal communication), but we were unable to demonstrate interaction *in vivo*. Our results show that repression by DAX-1 in a natural promoter context requires DNA binding (Fig. 5). DAX-1 defines a new class of nuclear receptors and extends the number of the responsive elements through which these factors exert their transcriptional activity. □

Methods

Protein purification and DNA-binding selection. Human DAX-1 was overexpressed in TB1 *E. coli* as a fusion protein with maltose-binding protein using a pMAL-c2 (New England Biolabs). Methods for DNA-binding selection will be described elsewhere. The sequences of the oligonucleotides used in this study are available upon request.

***In vitro* analysis of promoter sequences.** Plasmid DNA was incubated in 25 mM sodium cacodylate pH 8.5 and 10 mM MgCl₂ at 37 °C in the presence of 5 mM K₂Cr₂O₇ for 2 min. 2 μ l of 10% β -mercaptoethanol were added and DNA precipitated. DNA was amplified using ³²P-labelled primers for 1 min at 95 °C, 1 min at 60 °C and 30 s at 72 °C, for 15 cycles.

Cell transfection and luciferase assay. COS-1 cells were transfected using DEAE-dextran, Y-1 cells with calcium phosphate. *dax-1* promoter sequences were cloned in pGL2-enhancer (Promega). pGL1.3kb *StAR*¹⁴ was a gift from J. F. Strauss. In pGL1.3kb *StAR* DelH the sequence –61/27 in pGL1.3kb *StAR* is deleted. Standard luciferase assays were performed. DAX-1-expressing Y-1 cell lines were obtained using the expression plasmids pSG.DAX-1 (ref. 1) and pD383, which carries the neomycin-resistance gene under the control of the phosphoglycerate kinase promoter.

***In vivo* modification of plasmid DNA.** *dax-1* reporter transfected COS-1 cells were washed with PBS, incubated with 2% chloroacetaldehyde (CAA) in PBS for 1 min and lysed in 1 ml of 0.6% SDS, 10 mM EDTA. Extrachromosomal plasmid DNA was prepared and amplified using linear PCR in the presence of adequate ³²P-labelled primers for 1 min at 95 °C, 1 min at 60 °C and 30 s at 72 °C, for 35 cycles in a total volume of 20 μ l. PCR products were analysed on a 7% polyacrylamide denaturing gel.

Western and northern blot analyses. The monoclonal antibody 2F4 was used for DAX-1 detection⁵. *StAR* detection was performed on extracts from purified mitochondria¹¹. Northern blots were hybridized with a *StAR* full-length probe¹¹, using a GAPDH (glyceraldehyde-3-phosphate dehydrogenase) probe as control.

Steroid assay. Tissue culture supernatant from Y-1 clones was assayed for fluorogenic steroid content as described²³, using corticosterone (Sigma) as standard.

Received 29 July; accepted 6 October 1997.

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Acknowledgements. We thank E. Remboutsika, M. Lamas, B. Bardoni, J. F. Strauss III, K. L. Parker, G. Camerino and E. Borrelli for help, discussions, and gifts of material, and E. Heitz, Y. Lutz, B. Boulay and S. Metz for technical assistance. E.Z. is an EEC postdoctoral fellow; E.L. was supported by a Telethon Italy Fellowship. This study was funded by grants from the NIH (to D.M.S.) and from CNRS, INSERM, Centre Hospitalier Universitaire Régional, FRM, Rhône-Poulenc Rorer (Bioavenir) and ARC (P.S.-C.).

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corrections

The optical counterpart of the isolated neutron star RX J185635–3754

Frederick M. Walter & Lynn D. Matthews

Nature **389**, 358–360 (1997)

There was a typographical error in the declination of the optical counterpart given at the start of the fourth paragraph: this should be –37° 54' 35.8". □

Imaging of radiocarbon-labelled tracer molecules in neural tissue using accelerator mass spectrometry

R. E. M. Hedges, Z. X. Jiang, C. Bronk Ramsey, A. Cowey, J. D. B. Roberts & P. Somogyi

Nature **383**, 823–826 (1996)

We regret that we did not refer in this Letter to two publications^{1,2}. These authors showed that scanning SIMS (rather than SIAMS) can be used in a similar way to image tracers, including ¹⁴C, in tissue. We had not appreciated, from the published data, the extent to which the performance of the instrument might be equivalent to that of SIAMS. □

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Structure of the inhibitory receptor for human natural killer cells resembles haematopoietic receptors

Qing R. Fan, Lidia Mosyak, Christine C. Winter, Nicolai Wagtmann, Eric O. Long & Don C. Wiley

Nature **389**, 96–100 (1997)

The sixth sentence in the introductory bold paragraph should read: “The human p58 natural killer-cell inhibitory receptor clone 42 recognizes HLA-Cw4, -Cw2, -Cw5 and -Cw6, but not HLA-Cw3, -Cw1, -Cw7 or -Cw8, which are recognized by p58 killer-cell inhibitory receptor clone 43 (ref. 3).” □

Crystal structures of fragment D from human fibrinogen and its crosslinked counterpart from fibrin

Glen Spraggon, Stephen J. Everse & Russell F. Doolittle

Nature **389**, 455–462 (1997)

In a sentence on page 459 describing the orientation of the Gly-Pro-Arg-Pro-amide ligand as bound to the double-D fragment, the wrong aspartate was referred to on one occasion. The sentence should have read: “...the α-amino group being juxtaposed between the residues Asp₃₆₄ and Asp₃₃₀, and the guanidino group of the arginine lying between the carboxyl group of Asp₃₃₀ and the carboxamide of Gln₃₂₉ (Fig. 5a, b).” □

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Available either prestained or unstained, these ladders are affinity-purified, recombinant proteins that generate crisp, evenly spaced bands without background, minimizing 'holes' in the molecular weight. The ladder enables the real-time monitoring of the progress and quality of an electrophoretic separation, and can be used to assess the transfer of proteins when per-



The ladder to success may come from Gibco BRL's BenchMark SDS-PAGE ladder.

forming western blots. A proprietary coupling reaction creates uniformly labelled proteins for enhanced band sharpness. The large size range (10–220 K) enables the use of one ladder on a wide range of gel percentages, and standard 5- μ l loading yields bright bands on mini gels. Additional benefits of the product include triple-intensity reference bands on the unstained ladder for easy orientation; hot pink reference band on the prestained ladder for easy orientation; and the use of either silver or Coomassie Blue staining with the unstained ladder for precise size analysis.

Reader Enquiry No. 102

KinExA 3000

Sapidyne Instruments

Designed to measure affinity and kinetic constants of biological binding interactions, such as antibodies and antigens

The all new KinExA 3000 provides sensitivity for the measurement of affinity and kinetic constants to be measured over a



Here to affinity: the KinExA 300 seeks to avoid interference.

wider range: affinity constants of 10^2 to 10^{13} M $^{-1}$, K_d from 10^{-2} to 10^{-13} M, on-rates from 10^3 to 10^9 M s $^{-1}$, and off-rates from 10^{-6} to 10^0 s $^{-1}$. This provides measurement of the true solution-phase constants, and is completely free of interference inherent in other methods, such as mass transport effects.

Reader Enquiry No. 103

Polymorphprep

From Mediatech and Nycomed

High-quality, density-gradient media

Polymorphprep is well suited for the isolation of polymorphonuclear granulocytes (neutrophils, eosinophils) from whole blood. This is a solution of sodium metrizoate and dextran 500 with a density of 1.113 g ml $^{-1}$ and an osmolality of 450 mOsm. The protocol for the isolation of granulocytes using Polymorphprep along with details on blood cell separation can be found in the technical booklet *Centrifugation Techniques III: Isolation of Blood Cells*.

Reader Enquiry No. 104

Immobilized GST

From Pierce Chemical

This line of products uses a cross-linked, six per cent agarose for quick removal of anti-GST from target antibodies

Researchers should find immobilized GST useful for purifying target antibodies by subtractive affinity chromatography, helping to eliminate potential crossreactivity of endogenous GST-like proteins in the tissue under study. The immobilized GST comes in a 5-ml gel package or in 2 $\times$ 2-ml columns.

Reader Enquiry No. 105

Antibody arsenal

Active androstenedione EIA

From Metra Biosystems

Produced by Diagnostic System Laboratories, this is an enzyme immunoassay for direct measurement of androstenedione

The new EIA is a direct, non-isotopic assay that comes in a convenient 96-well microplate format and features Active-coated microplate technology. The addition of this assay to the range means that the company now offers a complete androgen assessment panel comprising RIAs and selected EIAs for testosterone, free testosterone, DHEA-S, SHBG and DHT. DSL claim to be unique in offering assays for direct DHEA and androstenediol glucuronide.

Reader Enquiry No. 106

Anti-human endoglin

From Dako

Clone SN6h is a monoclonal antibody against endoglin, a type I transmembrane protein

Highly expressed on human vascular endothelial cells of a large variety of tissues, endoglin expression has been demonstrated on non-T/non-B acute lymphoblastic leukaemia, acute myeloid, myelomonocytic and hairy-cell leukaemia cells. Dako's antibody against endoglin can be used on formalin-fixed, paraffin-embedded sections using high-sensitivity detection systems, such as the company's catalysed signal amplification (CSA) system and LSAB+ system. The antibody can also be used on cryostat sections and cell smears, as well as in western blots and immunoprecipitation techniques.

Reader Enquiry No. 107**Androgen receptor antibody**

From BioGenex

This monoclonal should be useful in the identification of androgen receptors in tissue sections of normal prostate, testis, female breast tissues, as well as cancers

This monoclonal androgen receptor antibody has been specifically selected to recognize a unique immunogenic N-terminal transactivation domain of the androgen receptor, which shows a relatively low degree of homology with other steroid receptors. The antibody (F39.4.1) can be used to stain formalin-fixed, paraffin-embedded androgen receptors in tissue sections. It is available in concentrated form or as a ready-to-use reagent optimized for use with the Super Sensitive biotin-streptavidin detection systems. F39.4.1 is currently available for research use only.

Reader Enquiry No. 108**Progesterone receptor antibody**

From Zymed

A highly specific antibody for staining of formalin-fixed, paraffin-embedded or frozen tissue sections

The new PgR clone, PR-2CS, should prove to be important in evaluating and treating of breast cancers. PgR is an estrogen regulating protein whose presence may indicate a responsive estrogen receptor pathway. It has been shown that such PgR-positive breast cancers characteristically show a good response to hormone therapy and thus may carry a better prognosis than PgR-negative cases. The antibody is available as a concentrate, prediluted or as a ready-to-use reagent for use with Zymed's second-generation Histostain-Plus (AEC or DAB) detection systems.

Reader Enquiry No. 109

Endoglin has expression on a variety of leukaemia cells, the SN6h clone is now offered by Dako.

Oestrogen receptor β polyclonal Ab

From ABR

A polyclonal antibody specific to the recently identified and cloned estrogen receptor β is now available

The antibody reacts with various cell types in normal and ER α knockout mice and normal rats. According to the manufacturer, it has been used successfully in immunohistochemistry, western blot and gel super-shift procedures. It is said to supershift rat ER- β specifically and efficiently, whereas no shift is observed with preimmune serum. The affinity-purified antibody is also available in a biotinylated form, expanding the immunostaining and immunoblotting strategies that are possible.

Reader Enquiry No. 110**Assays****Vect-RID kits**

From Universal Biologicals

For use in radial immunodiffusion (RID), an important laboratory procedure for the quantification of specific proteins in serum and other biological fluids

This product line is designed for the quantification of the immunoglobulins in the equine, bovine, canine, pig and feline families. Kits for the quantification of IgG in sheep, goat and llama are also available. Each kit contains all the materials to quantify selected serum proteins in certain animals, including three 16-well plates, standards, graph paper, report forms, procedure, a 5- μ l microdispenser and the Vet-RID reader (no dilution is necessary for normal samples).

Reader Enquiry No. 111**Histamine ¹²⁵I RIA**

From American Laboratory Products

To quantify histamine in urine, histamine release in peripheral lymphocytes and total histamine release in EDTA whole blood

In humans, histamine (β -imidazole ethylamine) is a potent mediator of anaphylaxis and is mostly found in the initial phase of the anaphylactic reaction. This RIA uses a competitive, double-antibody method, complete with six ready-to-use standards, ranging from 0 to 100 ng ml⁻¹, and low- and high-lyophilized controls. During the assay, derivitization of histamine to *N*-acetyl-

histamine is accomplished with a liquid acylation reagent. Analytical sensitivity of this method is a stated 0.1 ng ml⁻¹. The expected range for histamine in urine is 3 to 10 ng ml⁻¹. Heparin plasma standards and controls are also available upon request. This product is currently for research use only.

Reader Enquiry No. 112**Diastat anti-PR3 and anti-MPO assays**

From Shield Diagnostics

Three new ELISA kits for the detection of cathepsin G, lactoferrin and elastase in defining autoimmune disorders

The discovery of ANCA has revolutionized the diagnosis of patients with autoimmune diseases, such as vasculitis and glomerulonephritis. These kits join the Diastat anti-PR3 and anti-MPO assays for ANCA testing. Currently, most immunology laboratories

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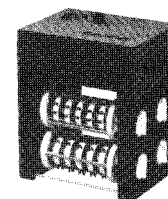
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READER ENQUIRY NO. 61

rely on perinuclear (p-ANCA) and cytoplasmic (c-ANCA) IFA patterns for diagnosis. The main target antigen for c-ANCA is proteinase 3 (PR3), while that for p-ANCA is myeloperoxidase (MPO). However, there is increasing evidence of a role for other antigens in ANCA testing; namely, cathepsin G, lactoferrin and elastase antibodies, which all contribute to atypical p-ANCA staining patterns. These x-ANCA, or 'snowstorm' patterns, have been reported in patients with SLE, rheumatoid arthritis, vasculitis and inflammatory bowel syndrome. Currently available for research use only, the kits will help classify samples with atypical patterns or samples positive for p-ANCA IFA, but with no evidence of anti-MPO antibodies.

Reader Enquiry No. 113

As quiet as a...

Mouse myeloid differentiation poster

From Serotec

The poster, Myeloid Differentiation in the Mouse, produced in conjunction with Immunology Today, is available free on request

The company's range of monoclonal anti-

bodies can be used to study mouse cell surface antigens, such as macrophage marker F4/80 and scavenger receptor 2F8. Certain reagents are also available in a wide range of formats, including FITC, RPE, RPE-Cy5 and biotin conjugates, as well as preservative-free formats for use in functional studies. In addition, there is a new range of matched pair antibodies for the measurement of mouse cytokines in ELISA. The Serotec range also includes human, rat, pig, sheep, cow, dog, cat, rabbit, guinea-pig, horse, monkey, chicken, turkey and goat reagents.

Reader Enquiry No. 114

TNF- α and sPLA₂ transgenic mice

From Taconic

Inflammatory mediator and arthritis animal models

TNF- α and sPLAs transgenic mice each contain a human gene for a key inflammatory mediator. TNF- α mice provide a genetic model of inflammatory arthritis, and sPLA₂ mice are useful for targeting human group II sPLA₂ inhibitors and performing basic research studies of in-

flammation and atherosclerosis. DNX Transgenics has developed both these models. TNF- α mice carry a 3'-modified human tumour necrosis factor (TNF) transgene and exhibit deregulated TNF expression and progressive development of severe inflammatory arthritis, without the need for experimental induction. Arthritis develops in 100 per cent of the mice with consistent onset. sPLA₂ mice express human group II secretory phospholipase A₂ (sPLA₂) transgene, resulting in expression of sPLA₂ serum levels eight times greater than non-transgenic control mice. The skin on these mice exhibits epidermal and adnexal hyperplasia, follicular hyperkeratosis and severe alopecia. Additionally, these mice are more susceptible to LPS-induced shock, have an altered lipoprotein metabolism and, when fed a high-cholesterol diet, are prone to develop atherosclerosis.

Reader Enquiry No. 115

These notes are compiled by Brendan Horton from information provided by the manufacturers. For more details, fill in the reader service card bound inside the journal.

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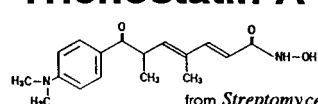
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